Studies on Role of Arachidonic Acid Metabolites and Mechanism of Their Formation during Activation of Human Platelets(Dissertation_全文)

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1993

CONTENTS

ABBREVIATIONS

$TXA_2(B_2)$: thromboxane A_2 (B_2)

STA_2 : 9,11-epithio-11,12-methano-TXA_2

ONO-3708: 9,11-dimethylmethano-11,12-methano-13,14-dihydro-13-aza-14-oxo-15-cyclopentyl-16,17,18,19,20-pentanor-15-epi-TXA_2

AA : arachidonic acid

PI : phosphoinositide(s) or phosphatidylinositol

PIP_2 : phosphatidylinositol 4,5-bisphosphate

DG : diacylglycerol

MG : monoacylglycerol

PA : phosphatidic acid

PE : phosphatidylethanolamine

PC : phosphatidylcholine

PLC : phospholipase C

PLA_2 : phospholipase A_2

EGTA : [ethylene bis(oxyethylenenitrilo)]tetraacetic acid

PMSF : phenylmethylsulfonyl fluoride

MES : 2-(*N*-morpholino)ethanesulfonic acid

PIPES : piperazine-N,N'-bis(2-ethanesulfonic acid)

HPLC : high-performance liquid chromatography

TLC : thin-layer chromatography

GENERAL INTRODUCTION

Platelets are activated by physiological stimuli, such as thrombin, collagen and ADP. Then, the platelets are aggregated via several biochemical events. The earliest event during the stimulation of human platelets is acceleration of phosphoinositide (PI) turnover. The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by PI-specific phospholipase C (PI-PLC) is a key process in the turnover (*1*). This hydrolysis is followed by the production of several second messengers (*2*). One of them, inositol 1,4,5-trisphosphate (IP_3) mobilizes Ca^{2+} from intracellular stores (*3*). It has demonstrated that IP_3 triggers Ca^{2+} mobilization in platelets activated with thrombin (*4*). Stimulation with collagen also elicited elevation of cytosolic free Ca^{2+} concentrations (*4*). However, in the presence of cyclooxygenase inhibitors such as indomethacin or aspirin, the rise in cytosolic free Ca^{2+} concentrations in collagen-activated platelets was suppressed (*5-7*). This suggests that arachidonic acid metabolites are involved in the elevation of cytosolic free Ca^{2+}. In this thesis, the author studied the role of arachidonic acid metabolites

and the mechanism of their formation during activation of human platelets. In Chapters I and II, the author demonstrated the role of thromboxane A_2, one of arachidonic acid metabolites, in the process of activation of human platelets with collagen. In Chapter III, the author revealed the mechanism of arachidonic acid release during activation of human platelets with collagen. Chapter IV describes the purification of PI-specific phospholipase C, which acts at the first step of arachidonic acid release, from human platelet cytosol.

CHAPTER I

ELEVATION OF CYTOSOLIC FREE Ca^{2+} IS DIRECTLY EVOKED BY THROMBOXANE A_2 IN HUMAN PLATELETS DURING ACTIVATION WITH COLLAGEN

One of the earliest biochemical changes associated with the coupling of agonists to platelet receptors is an increase in cytosolic Ca^{2+} concentration due to either the release of internally stored Ca^{2+} or influx from the extracellular medium. Inositol 1,4,5-trisphosphate (IP_3) triggers Ca^{2+} mobilization in platelets activated with thrombin (*4*). The increase in the cytosolic free Ca^{2+} concentration in collagen-activated platelets is suppressed in the presence of cyclooxygenase inhibitors (*5-7*), suggesting that arachidonic acid metabolites are involved in Ca^{2+} mobilization. However, the function of arachidonic acid metabolites, especially thromboxane A_2 (TXA_2) (*8*), during the signal transduction process remains unclear. This chapter describes that several nanomolar TXA_2 originating from inositol lipids (*9*) may directly cause Ca^{2+} mobilization without further activation of phospholipase C in human platelets during activation

with collagen.

MATERIALS AND METHODS

Materials. Aquasol-2 and [1-^{14}C]20:4 (54.9 mCi/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Collagen (Hormon-Chemie GmbH, München, Federal Republic of Germany), thrombin (Midorijuji, Osaka, Japan), fura-2/acetoxymethyl ester (Dojindo Laboratories, Kumamoto, Japan) were purchased from sources indicated. ONO-3708 and STA_2 were kindly gifted from Ono Pharmaceutical Co., Ltd. (Osaka, Japan). All other chemicals were of reagent grade and obtained from commercial sources.

Activation of Human Platelets by Thrombin, Collagen and STA_2. Blood was drawn from normal human volunteers using 13% of a solution containing 102 mM sodium citrate (tribasic), 15.6 mM citric acid, 17.7 mM sodium phosphate (dibasic), and 128.8 mM glucose as an anticoagulant. Platelet-rich plasma was obtained by centrifugation at 1100 x *g* for 5 min and concentrated 4-fold by centrifugation at 3800 x *g* for 5 min. The concentrated platelet-rich plasma was centrifuged at

100 x *g* for 10 min to remove red cells and incubated with [1-^{14}C]20:4 (10μCi/6x10^{10} platelets) at 25°C for 60 min or with 3 μM fura-2/acetoxymethyl ester at 37°C for 60 min. Platelets were washed twice with Tris/citrate/ bicarbonate buffer, pH 6.5 (*10*), containing 2 mM EDTA by centrifugation of the platelet-rich plasma at 1500 x *g* for 10 min. Washed platelets were suspended in Tris/citrate/bicarbonate buffer, pH 6.9 (*10*), without EDTA to a final concentration of 2x10^8 platelets/ml. Prior to activation of platelets, 5 mM $CaCl_2$ or 5 mM EGTA was added to the platelet suspension, and then platelet suspension was preincubated for 3 min at 37°C with or without 100 nM ONO-3708. The fura-2 loaded or [1-^{14}C]20:4 labeled human platelets were then activated with thrombin (2 units/ml), collagen (10 μg/ml) or STA_2 (various concentrations) with gentle stirring.

Measurement of Cytosolic Free Ca^{2+} Concentration----The ratio of the fluorescence intensities (emission, 500 nm) at the two excitation wavelengths, 340 and 380 nm, was continuously monitored with a Ca^{2+} analyzer (CAF-100; Japan spectroscopic Co., Ltd., Tokyo) simultaneously with aggregation. The

cytosolic free Ca^{2+} concentration was calculated using a dissociation constant for the fura-2-Ca^{2+} complex of 224 nM (*11*).

Extraction of Lipids----Human platelets ($2x10^8$/ml; 7.5 ml) labeled with [1-^{14}C]20:4 were stimulated with various concentrations of STA_2 in the presence of 5 mM $CaCl_2$. At 30 s after stimulation, the reaction was terminated by the addition of 15 ml of chloroform / methanol (1:2, v/v), containing 0.01% butylated hydroxytoluene and 0.1 mM tocopherol, and 7.5 ml of 2 M KCl containing 0.1 M EDTA. After shaking for 10 min, 15 ml of chloroform were added with vigorous shaking for an additional 10 min. After centrifugation at 3000 x g for 10 min, the chloroform layer was removed and the aqueous layer was extracted twice with 20 ml of chloroform. The combined chloroform fractions were dried under reduced pressure. The lipids were solubilized with 10 ml of chloroform and washed twice with 30 ml of water (*9*).

Separation of Lipid Classes----The lipid classes were separated by thin-layer chromatography. Plates were developed with chloroform/methanol/acetone/acetic

acid/water (100:50:100:4:10, v/v) at room temperature. The individual lipid classes were visualized by autoradiography. Diacylglycerol and phosphatidate fractions were scraped off from the plate and suspended in Aquasol-2/water/methanol (83:12:5, v/v) (*9*). The radioactivities of them containing [1-^{14}C]20:4 were counted by liquid scintillation counter.

RESULTS AND DISCUSSION

The basal free Ca^{2+} concentration in fura-2-loaded human platelets was 50 nM in the presence of 5 mM EGTA and 100 nM in the presence of 5 mM $CaCl_2$ (Fig. 1). This is in agreement with the results of Pollock and Rink (*12*). When $2x10^8$ fura-2-loaded platelets/ml were stimulated with 2 units thrombin/ml in the absence of extracellular Ca^{2+}, the cytosolic Ca^{2+} concentration increased from 50 to 300 nM with the release of internally stored Ca^{2+} (Fig. 1a). In the presence of external Ca^{2+}, the cytosolic Ca^{2+} concentration increased from 100 to 700 nM (Fig. 1b). The TXA_2 antagonist, ONO-3708 did not inhibit Ca^{2+} mobilization during activation with thrombin (Fig. 1c, d). When $2x10^8$ fura-2-loaded platelets/ml were stimulated with

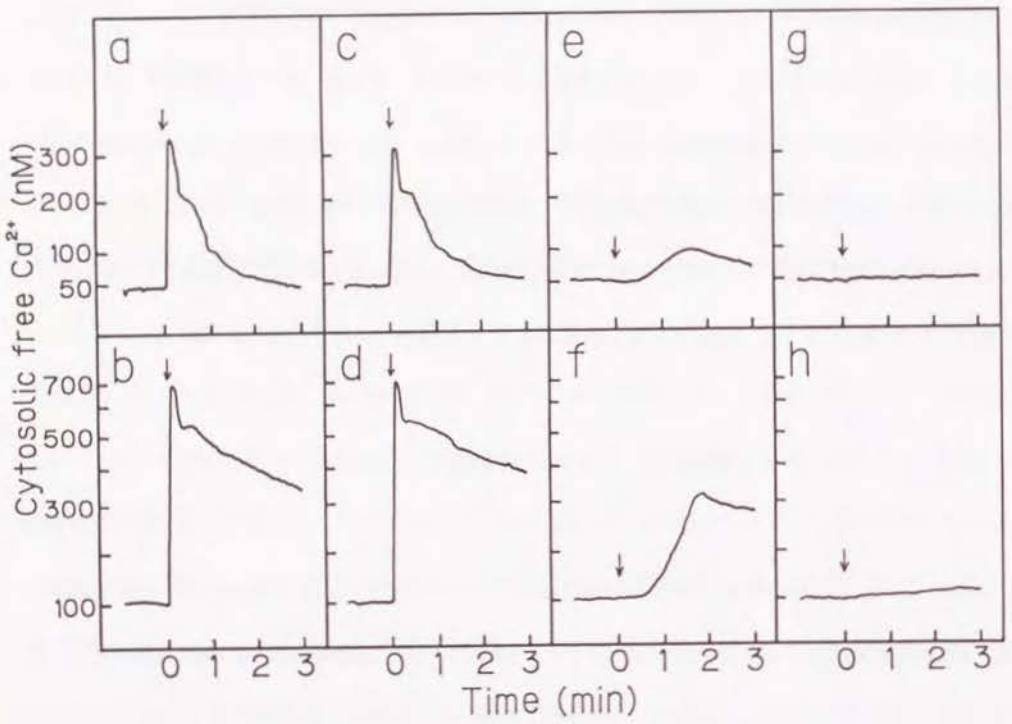

Fig. 1. Effect of a TXA_2 antagonist on the increase in cytosolic Ca^{2+} after stimulation of fura-2-loaded platelets with thrombin and collagen. The fura-2-loaded human platelets ($2x10^8$/ml) were suspended in the buffer (*10*) containing 5 mM EGTA or 5 mM $CaCl_2$, and the preincubated for 3 min at 37°C with or without 100 nM ONO-3708 prior to activation. They were then activated with thrombin (2 units/ml) or collagen (10 µg/ml) with gentle stirring. The calibration scale for cytosolic free Ca^{2+} is shown on the vertical axis. The traces shown are representative of triplicate determinations. Stimulation with thrombin with 5 mM EGTA (a) or 5 mM $CaCl_2$ (b), stimulation with thrombin in the presence of 100 nM ONO-3708 with 5 mM EGTA (c) or 5 mM $CaCl_2$ (d), stimulation with collagen with 5 mM EGTA (e) or 5 mM $CaCl_2$ (f), and stimulation with collagen in the presence of 100 nM ONO-3708 with 5 mM EGTA (g) or 5 mM $CaCl_2$ (h). The agonists were added at the times indicated by arrows.

10 µg collagen/ml in the absence of extracellular Ca^{2+}, the cytosolic Ca^{2+} concentration increased from 50 to 100 nM with the release of internally stored Ca^{2+} (Fig. 1e), and shape changes and ATP secretion began (data not shown) after a lag period of 30 s. Collagen evoked an increase in cytosolic Ca^{2+}, from 100 to 300 nM, after a lag period of about 30 s in the presence of extracellular Ca^{2+} (Fig. 1f). Contrary to our results, it has been proposed that collagen can stimulate platelets without raising the cytosolic free Ca^{2+} concentration (*6,13*). This discrepancy may be due to technical limitations of the use of quin2 for determining small increases in cytosolic free Ca^{2+}, since fluorescence intensity measurements by the quin2 method are disturbed by the scattering of light caused by collagen itself (data not shown). ONO-3708 completely inhibited the increase in cytosolic Ca^{2+} (Fig. 1g, h), shape changes and ATP secretion (data not shown). Similar results were obtained with another TXA_2 antagonist, ONO-11120. This indicates that TXA_2 receptor occupation (*14,15*) is closely linked to the elevation of cytosolic free Ca^{2+} in human platelets.

Instead of TXA_2, which has a very short life, a stable TXA_2 mimetic, STA_2 was used, which caused a

rapid increase in the cytosolic Ca^{2+} concentration through either release from internal stores or influx from the external medium without a lag period. Maximal and half-maximal Ca^{2+} release required approximately 20 and 3 nM STA_2, respectively (Fig. 2). With regard to Ca^{2+} influx, similar values were obtained. However, maximal and half-maximal activation of phospholipase C required about 100 and 18 nM STA_2, respectively (Fig. 3). Pollock *et al.* (*14*) reported, with a prostaglandin H_2 analog, 9,11-epoxymethanoprostaglandin H_2, that maximal and half-maximal Ca^{2+} influx were observed at agonist concentrations of 1000 and 80 nM, respectively. These values were close to those for phosphatidate formation. This is not in agreement with my results. The discrepancy may be due to the structural differences between the agonists.

Half-maximal Ca^{2+} release requires about 1 μM IP_3 (*16*). TXA_2/endoperoxide receptors were reported to be linked to phospholipase C activation and IP_3 production (*14,17*), in order to reinforce the effect of collagen. However, the STA_2 concentrations required for half-maximal Ca^{2+} release (3 nM) and release+influx (4 nM) are lower than that for the activation of phospholipase

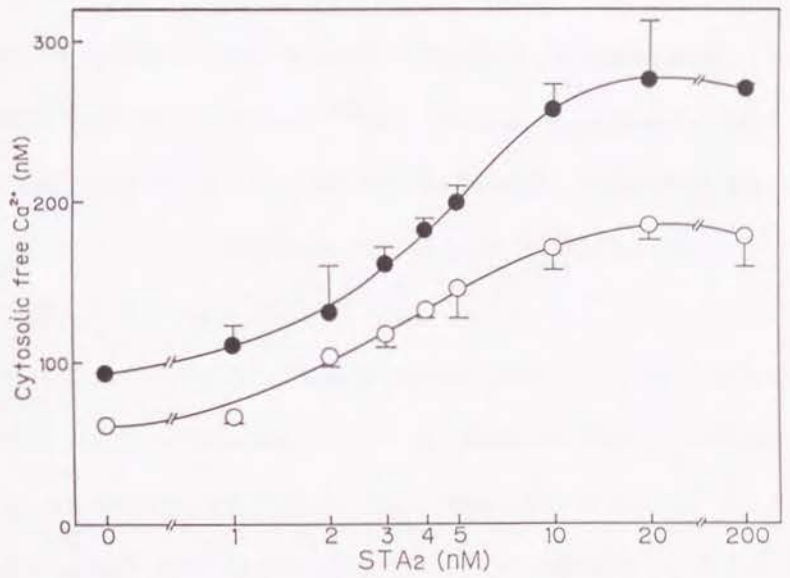

Fig. 2. Effect of the STA_2 concentration on the Ca^{2+} release from internal stores and Ca^{2+} influx. Fura-2-loaded platelets ($2x10^8$/ml) were suspended in the Tris-citrate-bicarbonate buffer, pH 6.9, containing 5 mM EGTA (◯) or 5 mM $CaCl_2$ (●), and then stimulated with various concentrations of STA_2. Data are presented as means ± S.D. (n=3).

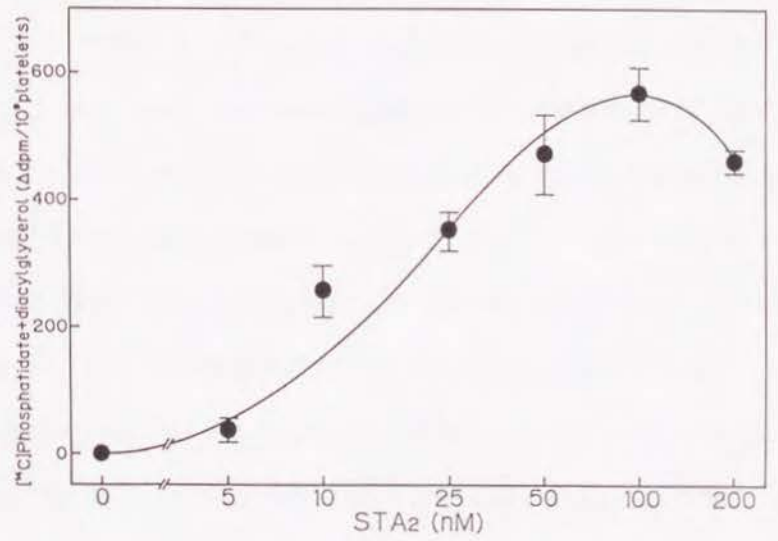

Fig. 3. Effect of the STA_2 concentration on the activation of phospholipase C. Human platelets ($2x10^8$/ml) labeled with [1-^{14}C]arachidonic acid were stimulated with various concentrations of STA_2 in the presence of 5 mM $CaCl_2$. Data (phosphatidate + diacylglycerol) are presented as means ± S.D. (n=3).

C (18 nM). Therefore, it is likely that TXA_2-receptor occupancy can directly cause Ca^{2+} mobilization without activation of phospholipase C.

CHAPTER II

DIHYDROPYRIDINE-SENSITIVE AND INSENSITIVE Ca^{2+} CHANNELS IN HUMAN PLATELETS

Receptor-operated elevation of the cytosolic Ca^{2+} level can be caused by release from intracellular stores and influx from the extracellular fluid, which is activated by inositol 1,4,5-trisphosphate (IP_3) and its derivatives (*18,19*). Thromboxane A_2 (TXA_2), a product of agonist-induced lipid metabolism in human platelets, activates phospholipase C, which then produces IP_3 (*14,17*). However, Chapter I described that the TXA_2 analogue, 9,11-epithio-11,12-methano-TXA_2 (STA_2), at low concentrations mobilized Ca^{2+} without the activation of phospholipase C in human platelets, suggesting the presence of Ca^{2+} channels other than those mediated by IP_3. This chapter describes the presence of dihydropyridine-sensitive and insensitive Ca^{2+} channels in human platelets and that TXA_2 may activate dihydropyridine-sensitive Ca^{2+} channels.

MATERIALS AND METHODS

Materials----cAMP[^{125}I] radioimmunoassay kit was purchased from Amersham. Nifedipine, nicardipine, diltiazem and tetraethylammonium were purchased from Sigma (St. Louis, MO, U.S.A.). All other materials were obtained as described in "MATERIALS AND METHODS" of Chapter I.

Measurement of Cytosolic Free Ca^{2+} Concentration----Cytosolic free Ca^{2+} concentration was measured as described in "MATERIAL AND METHODS" of Chapter I.

RESULTS AND DISCUSSION

In collagen-activated human platelets, elevation of the cytosolic Ca^{2+} level was not caused by collagen itself, but by TXA_2, which was produced via collagen-induced inositol lipid metabolism (*9*). Nifedipine, a dihydropyridine derivative, specifically blocks the L-type (*20*) voltage-dependent Ca^{2+} channels of vascular smooth muscle (*21*). This drug inhibited both the Ca^{2+} influx and release from internal stores in the early

stage of activation of human platelets by collagen (Fig. 4a, c). Nifedipine also inhibited Ca^{2+} mobilization caused by 10 nM STA_2, a concentration at which phospholipase C was slightly activated (Fig. 4b, d). In the presence of 5 mM EGTA, the STA_2-induced increase in cytosolic free Ca^{2+} decreased by 24 % (Fig. 4b, d), indicating that the increase in free Ca^{2+} is

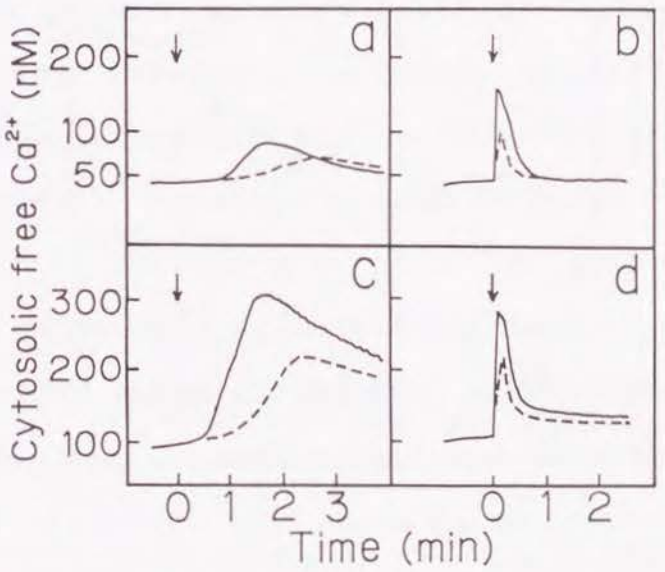

Fig. 4. Inhibition by nifedipine of elevation of the cytosolic Ca^{2+} level in fura-2 loaded human platelets stimulated with collagen or STA_2. The fura-2-loaded human platelets ($2x10^8$/ml) were suspended in the buffer (*10*) containing 5 mM EGTA or 5 mM $CaCl_2$, and then incubated for 3 min at 37°C with or without inhibitors prior to activation. They were then activated with collagen (10 µg/ml) or STA_2 with gentle stirring. Stimulation of fura-2 loaded human platelets ($2x10^8$/ml) was performed with collagen (10 µg/ml) (a,c) or 10 nM STA_2 (b,d) in the presence of 5 mM EGTA (a,b) to examine Ca^{2+} release from internal stores or 5 mM $CaCl_2$ (c,d) to examine Ca^{2+} influx and release from internal stores. Prior to stimulation, fura-2 loaded platelets were incubated for 3 min with (broken lines) or without (solid lines) 25 µM nifedipine. The agonists were added at time 0 (arrows).

due mainly to release from internal stores rather than influx, although Ca^{2+} influx preceded Ca^{2+} release from internal stores (*22*). Nifedipine-inhibition curves, with ID_{50} of 20 μM, were obtained with and without extracellular Ca^{2+} (Fig. 5a, b). The mode of inhibition by nifedipine was noncompetitive as to STA_2 (Fig. 5c, d). Nicardipine, another dihydropyridine derivative, also inhibited the elevation of cytosolic Ca^{2+}. Diltiazem, a benzothiazepine derivative, inhibited the Ca^{2+} influx and release from internal stores caused by 10 nM STA_2, ID_{50} being 350 and 150 μM, respectively (Fig. 6).

Thus the ID_{50} values are higher than those in the case of smooth muscle (*21*). Hence, it seems unlikely that a specific channel blockade is occurring. However, stimulus-secretion systems are known to be less sensitive to Ca^{2+} antagonists than smooth and cardiac muscles (*21*). The inhibition by these Ca^{2+} blockers was abolished on washing of the platelets with the buffer containing no Ca^{2+} blockers. Tetraethylammonium (2 mM), a M-channel blocker, did not inhibit the Ca^{2+} mobilization by STA_2. The cytosolic Ca^{2+} level is regulated by cAMP (*23*). The levels of cAMP in human platelets 0, 5, 10 and 30 s after the addition

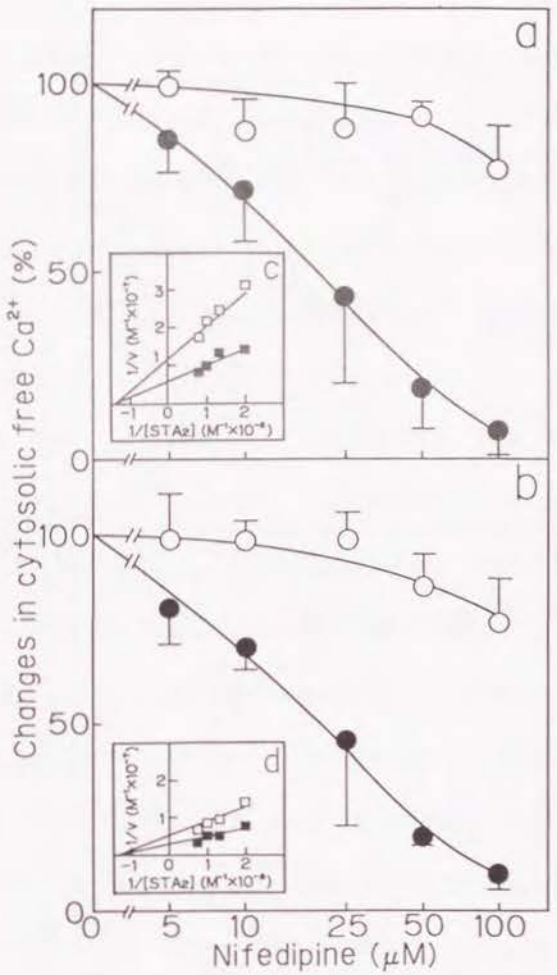

Fig. 5. Effect of nifedipine on elevation of the cytosolic free Ca^{2+} level in fura-2-loaded platelets stimulated with STA_2 or thrombin in the presence and absence of extracellular Ca^{2+}. Fura-2-loaded platelets ($2x10^8$/ml) were stimulated with 10 nM STA_2 (●) or thrombin (1 unit/ml) (○) in the presence of 5 mM EGTA (a) or 5 mM $CaCl_2$ (b). With thrombin stimulation, 1 uM ONO-3708, a TXA_2 antagonist, was added to exclude the effect of TXA_2. Prior to stimulation, the platelets were incubated with various concentrations of nifedipine for 3 min. The changes in free Ca^{2+} without nifedipine (110 or 145 nM with 5 mM EGTA or 5 mM $CaCl_2$ for STA_2, and 223 or 506 nM with 5 mM EGTA or 5 mM $CaCl_2$ for thrombin) were regarded as 100 %. The plots of 1/v against $1/[STA_2]$ with (□) or without (■) 20 μM nifedipine are shown (c, d), where v is the Ca^{2+} increase at 10 s after STA_2 addition. The data are presented as means±S.D. for 3 experiments, using platelets from different volunteers.

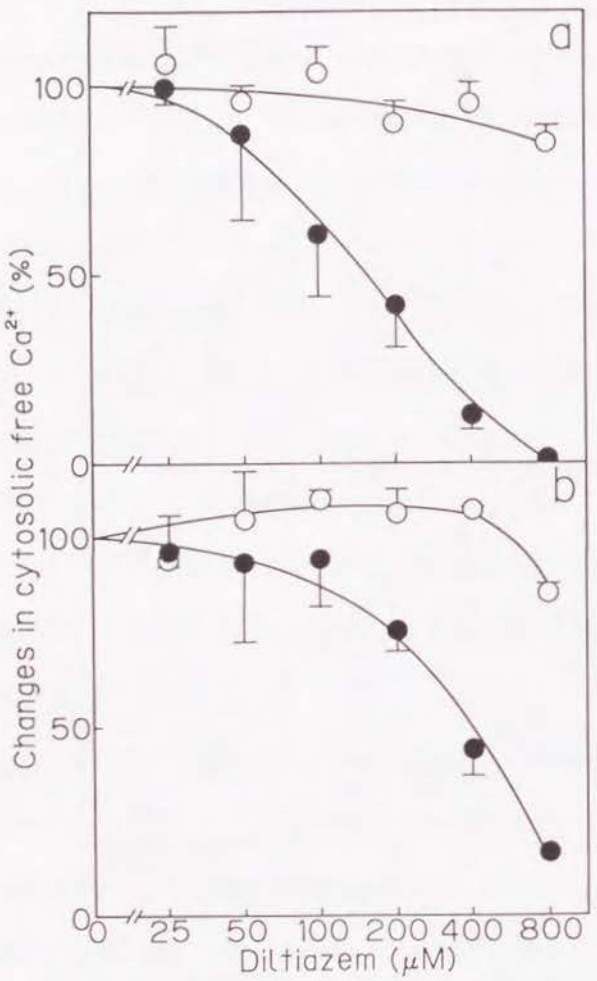

Fig. 6. Effect of diltiazem on elevation of the cytosolic free Ca^{2+} level in fura-2-loaded platelets stimulated with STA_2 or thrombin in the presence and absence of extracellular Ca^{2+}. The assay conditions were the same as in the legend to Fig. 5, except that diltiazem was used instead of nifedipine.

of 10 nM STA_2 were determined by means of a radioimmunoassay to be 1.43±0.12, 1.83±0.20, 1.87±0.23 and 1.66±0.31 pmol/10^8 platelets (mean±S.D., n=4) in the presence of 50 μM nifedipine, and 1.13±0.17, 1.35±0.18, 1.50±0.22 and 1.39±0.17 pmol/10^8 platelets (mean±S.D., n=4) in the absence of the inhibitor, respectively. Thus, nifedipine did not cause elevation of the cAMP level to one that affect cytosolic free Ca^{2+}. From these results, it appears likely that TXA_2-operated Ca^{2+} channels are present in plasma and organelle membranes of human platelets with lower affinities to Ca^{2+} blockers than those in the cases of vascular smooth and cardiac muscles.

Nifedipine and diltiazem did not inhibit Ca^{2+} influx or release from internal stores in thrombin-stimulated human platelets in the presence of the TXA_2 antagonist, ONO-3708, which was added to exclude TXA_2-dependent Ca^{2+} mobilization (Figs. 5 and 6). The results obtained with diltiazem are in agreement with those obtained by Doyle and Rüegg (*24*). It was noted that nifedipine and diltiazem did not inhibit the elevation of the cytosolic Ca^{2+} level caused by a high concentration of STA_2 (100 nM), which was sufficient to activate phospholipase C. In collagen-activated

platelets, the degree of nifedipine inhibition gradually decreased with time (Fig. 4a, c). This may be due to activation of phospholipase C with the time-dependent increase in TXA_2 (*9*). Therefore, in human platelets stimulated with thrombin (*4*) and high concentrations of STA_2, IP_3 functions to release Ca^{2+} from internal stores through voltage-insensitive channels like the receptor-operated Ca^{2+} channels detected in T-cells (*19*), since the channels regulated by IP_3 are insensitive to Ca^{2+} blockers.

The presence of dihydropyridine-susceptible structures in human platelets was reported by Erne *et al.* (*25*), although there are no known natural agonists for these structures. However, these structures may differ from the Ca^{2+} channels mediated by low concentrations of STA_2, since the two structures show different affinities to nifedipine. The presence of two distinct, dihydropyridine-sensitive and insensitive, Ca^{2+} channels dependent on the concentrations and classes of agonists in human platelets will greatly increase our understanding of the mechanism of platelet activation.

CHAPTER III

THE MECHANISM OF ARACHIDONIC ACID RELEASE IN COLLAGEN-ACTIVATED HUMAN PLATELETS

When human platelets are stimulated with various agonists such as thrombin or collagen, the platelets are aggregated via several biochemical events. The earliest event during the stimulation of human platelets is acceleration of phosphoinositide (PI) metabolism. The initial reaction of the metabolism is the hydrolysis of phosphoinositides by PI-specific phospholipase C (PI-PLC) (*4*). Inositol 1,4,5-trisphosphate (IP_3), one of the reaction products mobilizes ionized calciums from intracellular stores as second messenger (*26*). IP_3 triggers Ca^{2+} mobilization in platelets activated with thrombin (*16*). Stimulation with collagen also elicited elevation of cytosolic free Ca^{2+} concentrations (*5*). However, in the presence of cyclooxygenase inhibitors such as indomethacin or aspirin, the rise in cytosolic free Ca^{2+} concentrations in collagen-activated platelets was suppressed (*6*). This suggests that collagen itself cannot evoke an increase in cytosolic free Ca^{2+} and

that arachidonic acid (AA) metabolites are involved in the elevation of cytosolic free Ca^{2+}. CHAPTER I demonstrated that several nanomolar of thromboxane A_2 (TXA_2) directly caused Ca^{2+} mobilization without further activation of PI-PLC during activation with collagen. Hence, it is likely that TXA_2 plays an important role for elevation of cytosolic free Ca^{2+} in collagen-activated human platelets. The rate-limiting step in the process of TXA_2 formation is the release of AA from membrane phospholipids. PI was hydrolyzed to supply free AA in collagen-activated human platelets in the absence of extracellular Ca^{2+} and other phospholipids were degraded in the presence of extracellular Ca^{2+} (9). In the absence of extracellular Ca^{2+}, cytosolic free Ca^{2+} concentrations were elevated from 50 nM to about 100 nM. Whereas, cytosolic free Ca^{2+} concentration was elevated to about 300 nM in the presence of extracellular Ca^{2+}. This suggests that concentrations of cytosolic free Ca^{2+} determine the degradation of phospholipid classes which supply AA to cytosol. In this chapter, the author attempted to identify the pathway(s) responsible for AA release at the early stage of stimulation and reveal the mechanism of AA release in connection with

cytosolic free Ca^{2+} concentrations during activation of human platelets with collagen.

MATERIALS AND METHODS

Materials. Phosphatidylethanolamine plasmalogen (bovine brain) was purchased from Serdary research laboratory. Aquasol-2, [1-^{14}C]stearic acid, [1-^{14}C]arachidonic acid, [^{3}H]arachidonic acid and TXB_2 radioimmunoassay kit were from Du Pont-New England Nuclear (Boston, MA, U.S.A.). Carrier-free [^{32}P]Pi was from the Japan Radioisotope Association (Tokyo, Japan). Leupeptin was from the Peptide Institute (Osaka, Japan). Heptyl-β-D-thioglucoside and fura-2 acetoxymethyl esters were from Dojindo Laboratories (Kumamoto, Japan). Collagen was from Hormon-Chemie (Munchen, Germany). The TXA_2 antagonist, ONO-3708 (9,11-dimethylmethano-11,12-methano-13,14-dihydro-13-aza-14-oxo-15-cyclopentyl-16,17,18,19,20-pentanor-15-epi-TXA_2) and the TXA_2 analogue, STA_2 (9,11-epithio-11,12-methano-TXA_2) were gifts from Dr. A. Kawasaki of the Research and Development Division, Ono Pharmaceutical Co., Ltd. (Osaka,Japan). Phospholipase C (grade I, from *Bacillus cereus*) was purchased from

Boehringer Mannheim. All other chemicals were of reagent grade and obtained from commercial sources.

Preparation of human platelets. Blood was drawn from normal human volunteers into a 13% solution of 102 mM sodium citrate (tribasic), 15.6 mM citric acid, 17.7 mM sodium phosphate (dibasic), and 128.8 mM glucose as an anticoagulant. Platelet-rich plasma was obtained by centrifugation at 1,100 x *g* for 5 min, followed by a 4-fold concentration by centrifugation at 3,800 x *g* for 5 min. The concentrated platelet-rich plasma was then centrifuged at 100 x *g* for 10 min to remove red cells. For loading of fura-2, the concentrated platelet rich plasma was incubated with 3 μM fura-2 acetoxymethyl ester at 37 ^{o}C for 60 min. For [^{32}P]Pi labeling, the concentrated platelet-rich plasma was incubated with [^{32}P]Pi (2 mCi/20 ml) at 37 ^{o}C for 60 min. For [^{14}C]arachidonic acid labeling, the concentrated platelet-rich plasma was incubated with [^{14}C]arachidonic acid (0.75 μCi/45 ml) at 25 ^{o}C for 60 min. After these loading or labeling, platelets were washed twice by centrifugation at 1500 x *g* for 10 min with Tris-citrate-bicarbonate buffer (*10*), pH 6.5 containing 2 mM EDTA. Washed platelets were suspended

in Tris-citrate-bicarbonate buffer (*10*), pH 6.9 without EDTA.

Measurement of cytosolic free Ca^{2+} concentrations. Fura-2 loaded human platelets (2 x 10^{8} /ml) prepared as described above were preincubated for 1 - 3 min at 37 ^{o}C with or without ONO-3708 (0.1 μM) prior to activation. They were then activated with collagen (10 μg/ml) or various concentrations of A23187 with gentle stirring. The ratio of fluorescence intensities (500 nm,emission) at two excitation wavelengths of 340 and 380 nm was continuously monitored with a Ca^{2+} analyzer (CAF-100, Japan Spectroscopic Co., Ltd.). The cytosolic free Ca^{2+} concentration was calculated by the use of dissociation constant for the fura-2-Ca^{2+} complex of 224 nM (*11*).

Analysis of phospholipid metabolism. ^{32}Pi-labeled platelet suspension (2 x 10^{8}/ml) was preincubated with 5 mM EGTA for 3 min at 37 ^{o}C and activated with collagen (10 μg/ml). Aliquots (1 ml) were removed at various times and mixed with 3.6 ml of chloroform : methanol : conc. HCl (100:200:2, v/v). The phase was separated by adding 1.2 ml of chloroform

and 1.2 ml of 2 M KCl/0.1 M EDTA. The lower organic layer was removed and the aqueous layer was washed twice with 2.5 ml of chloroform. The combined organic layer was dried under N_2 flow. The lipids were separated on silica gel G plates (Merck) using chloroform : methanol : 4 N NH_4OH (45:35:10, v/v). After drying *in vacuo* for 1 h at room temperature, the plates were redeveloped with the same solvent in the same direction. The lipids were visualized by autoradiography, scrapped off from the plate and counted by liquid scintillation in Aquasol-2 : water : methanol (83:12:5, v/v).

Analysis of arachidonic acid metabolism. [^{14}C]arachidonic acid-labeled platelet suspension (2 x 10^8/ml; 7.5 ml) was incubated with 5 mM $CaCl_2$ and 0.1 μM ONO-3708 for 3 min at 37 ^{o}C. Then, collagen (10 μg/ml) or vehicle was added into platelet suspension, followed by stimulation with various concentrations of A23187. After 3 min, the reaction was terminated by adding 15 ml of chloroform : methanol (1:2, v/v) containing 0.01 % BHT and 0.1 mM α-tocopherol and 7.5 ml of 2 M KCl/0.1 M EDTA. After shaking for 10 min, 15 ml of chloroform was added, followed by shaking

again for 10 min. After centrifugation, upper aqueous layer was separated and washed twice with chloroform. The combined organic layer was dried under N_2 flow. The lipids were separated on silica gel G plates (Merck) using chloroform : methanol : acetone : acetic acid : water (100:50:100:4:10, v/v). The AA metabolites and AA containing lipids were visualized by autoradiography and counted as described above. The sum of radioactivities corresponding to hydroxyheptadecatrienoic acid, hydroxyeicosatetraenoic acid and TXB_2 was counted as production of AA metabolites. To examine the formation of AA metabolites in STA_2-stimulated human platelets, various concentrations of STA_2 were added into platelet suspension (2 x 10^8/ml; 7.5 ml). After 30 sec or the indicated time, produced AA metabolites were measured as described above. For determination of TXB_2, TXB_2 radioimmunoassay kit was used.

Preparation of crude DG lipase and MG lipase sample. Washed human platelets prepared as described above without loading or labeling were suspended in the lysis buffer [20 mM Tris/HCl (pH 7.4), 20 mM EGTA , 0.5 mM leupeptin, 1 mM dithiothreitol] and sonicated twice

with a Branson sonicator model 250 at setting 3 for 30 sec. The sonicated platelets were centrifuged at 200,000 x *g* for 1 hr and then separated to cytosolic fraction and membrane fraction. The resultant membrane fraction was solubilized by solubilizing buffer (20 mM Tris/HCl, pH 7.4, 5 mM EDTA, 0.5 mM PMSF, 1 mM dithiothreitol, 30 % glycerol, 2 % heptyl-β-D-thioglucoside) with gentle stirring for 60 min at 4 ^{o}C. The solubilized fraction was obtained by centrifugation under the same conditions described above and was used as enzyme sources.

Preparation of labeled substrates. 1-[^{14}C]Stearoyl-diacylglycerol was prepared as described below (Fig.7). 2-Acyllysophosphatidylethanolamine was generated from phosphatidylethanolamine plasmalogen by acid treatment as incubation in 0.05 N HCl, 0.1 M H_3BO_3 / ethanol for 2 hr at 37 ^{o}C. The generated 2-acyllysophosphatidylethanolamine was isolated by thin-layer chromatography using chloroform : methanol : acetone : acetic acid : water (100:50:100:4:10, v/v). The resultant 2-acyllysophosphatidylethanolamine was extracted from scrapped TLC powder with chloroform : methanol (2:1, v/v). 1-[^{14}C]Stearoylphosphatidyl-

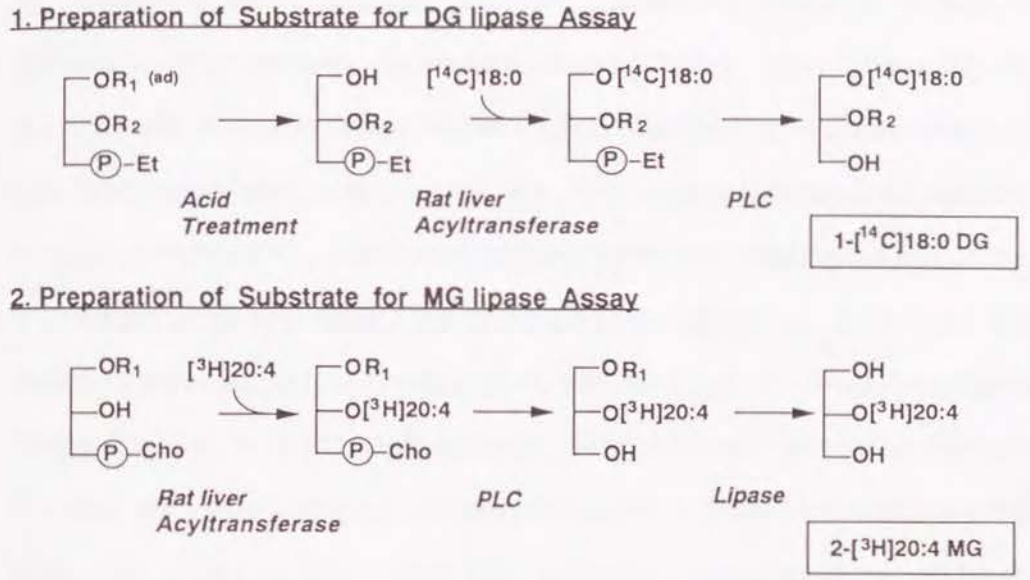

Fig. 7. Preparation of labeled substrates.
Schematic representation of the preparing methods of labeled substrates for DG lipase and MG lipase assays. See "MATERIALS AND METHODS" for explanation. R, acyl chain; Ⓟ, phosphate group; Et, ethanolamine; Cho, choline; 18:0, stearic acid; 20:4, arachidonic acid; PLC, phospholipase C.

ethanolamine was prepared biosynthetically from rat liver microsomes using [^{14}C]stearic acid and 2-acyllysophosphatidylethanolamine according to Lands and Merkl (27). 1-[^{14}C]Stearoyldiacylglycerol was obtained by treating 1-[^{14}C]stearoylphosphatidyl-ethanolamine with phospholipase C (*Bacillus cereus*) for 3 hr at 30 ^{o}C. The obtained labeled diacylglycerol

was dissolved in ethanol and stored at -20 ^{o}C to prevent the isomerization of 2- to 1-acyl migration to a minimum. To prepare a labeled substrate for MG lipase assay, 1-stearoyl-2-[^{3}H]arachidonoylphosphatidylcholine was biosynthesized as described above using 1-stearoyl-2-lysophosphatidylcholine and [^{3}H]arachidonic acid. The labeled phospholipid was hydrolyzed by phospholipase C as mentioned above. The resultant labeled diacylglycerol was suspended in 1.1 ml of 0.18 M Tris-HCl buffer (pH 6.5) containing bovine serum albumin (2.27 mg/ml), $CaCl_2$ (18 mM), H_3BO_3 (18 mM) and Triton X-100 (0.145 mg/ml). The suspension was dispersed by sonication for 1 min twice at 4 ^{o}C. After sonication, 0.9 ml of pancreatic lipase (Sigma, 0.1 mg/ml) was added to the suspension and incubated for 30 min at 37 ^{o}C with gentle shaking (*28*). The reaction was terminated by adding 4 ml of chloroform : methanol (2:1, v/v) and 0.2 ml of conc. HCl. After shaking by hand, phases were separated by centrifugation. The upper phase was washed twice with 2.6 ml chloroform. The lower phases were combined, dried under N_2 flow and dissolved in ethanol. The obtained 2-[^{3}H]arachidonoylmonoacylglycerol was kept at -20 ^{o}C (Fig. 7).

Enzyme assays. DG lipase assay was performed as follows. The radiolabeled diacylglycerol (approx. 40,000cpm = 10nmol per one assay) was transferred into 80 µl of 50 mM MES-NaOH, pH 7.0, followed by vortex mixing and sonicated twice with a Branson sonicator model 250 at setting 8 for 1 min. The reaction was started by mixing with the enzyme source (20µl). After incubation for 60 min at 37 ^{o}C, the reaction was terminated by the addition of 1.5 ml of chloroform/ methanol / heptane (125:140:100, v/v) (*29*). Then, stearic acid (20 µl of 25 mM soln.) as carrier and 0.5 ml of K_2CO_3-$KHCO_3$(pH 10.0) were added and mixed with vortex for 10 sec. The phase separation was performed by centrifugation and aliquots of the upper layer were used for scintillation counting in 4 ml of Aquasol-2. MG lipase assay was performed under the same conditions described for DG lipase assay using radiolabeled monoacylglycerol (approx. 10,000cpm = 10nmol per assay) and 25 mM arachidonic acid was used as substrate and carrier, respectively. Various free Ca^{2+} concentrations were obtained by using Ca^{2+}-EGTA buffers (pH 7.0) containing 2 mM EGTA (final concentration) and the appropriate amount of $CaCl_2$ (*30*).

Phospholipid metabolism during activation of human platelets with collagen.

The formation of PA, lysoPA and lysoPI in collagen-activated human platelets was measured in the absence of extracellular Ca^{2+}. PA was increased in a time-dependent manner, whereas lysoPA and lysoPI were not formed under this condition (Fig. 8A). The formation of TXB_2 increased in parallel with the formation of PA (Fig. 8B).

Effects of Ca^{2+} on DG lipase and MG lipase activities.

It was investigated whether MG and DG lipases could be activated under the low Ca^{2+} condition. These enzymes were solubilized from microsome fraction of human platelets. About 70-80 % of total activities and about 30 % of total proteins of the crude membrane fraction were solubilized by 2 % heptyl-β-D-thioglucoside (data not shown). Then, effects of Ca^{2+} concentrations on the solubilized enzymes were measured. As shown in Fig. 9, DG lipase activity was not significantly affected in the presence of various Ca^{2+} concentrations, whereas MG lipase

activity was slightly affected by Ca^{2+}. The hydrolytic activity of MG lipase was maximal at 10^{-7}-10^{-6} M Ca^{2+} concentrations, while higher Ca^{2+} concentrations showed rather an inhibitory effect.

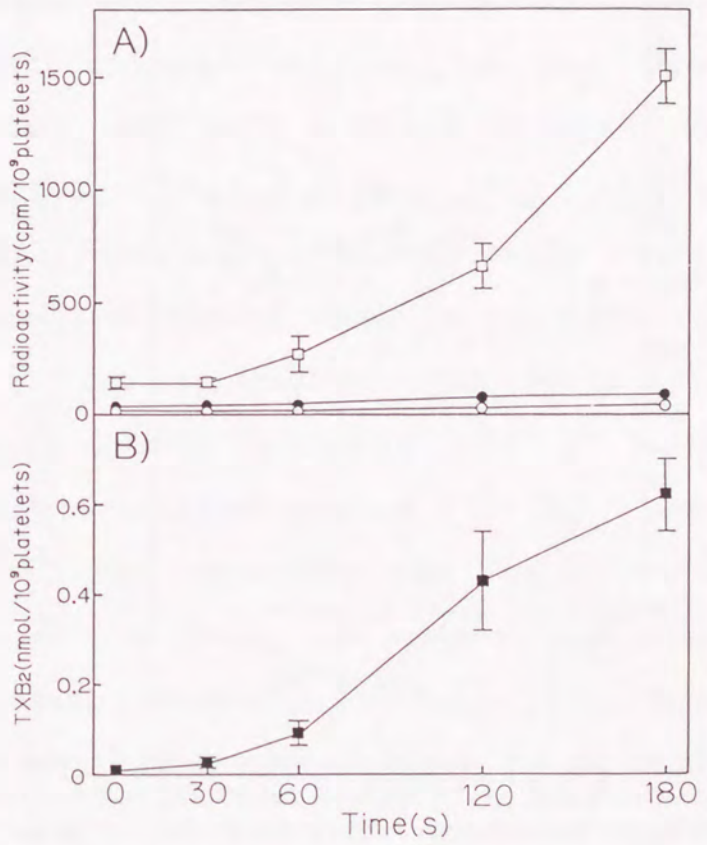

Fig. 8. Lipid metabolism during stimulation of human platelets with collagen in the absence of extracellular Ca^{2+}.
A. [^{32}P]Pi-labeled platelets were stimulated with 10 μg/ml collagen in the presence of 5 mM EGTA. At appropriate intervals, aliquots were removed and analyzed as described in the "MATERIALS AND METHODS". (□), PA; (●), lysoPA; (◯), lysoPI.
B. Cold platelets were stimulated as described above. At appropriate intervals, aliquots were removed and TXB_2 were determined as described in the "MATERIALS AND METHODS". Data are given as mean ± S.D.

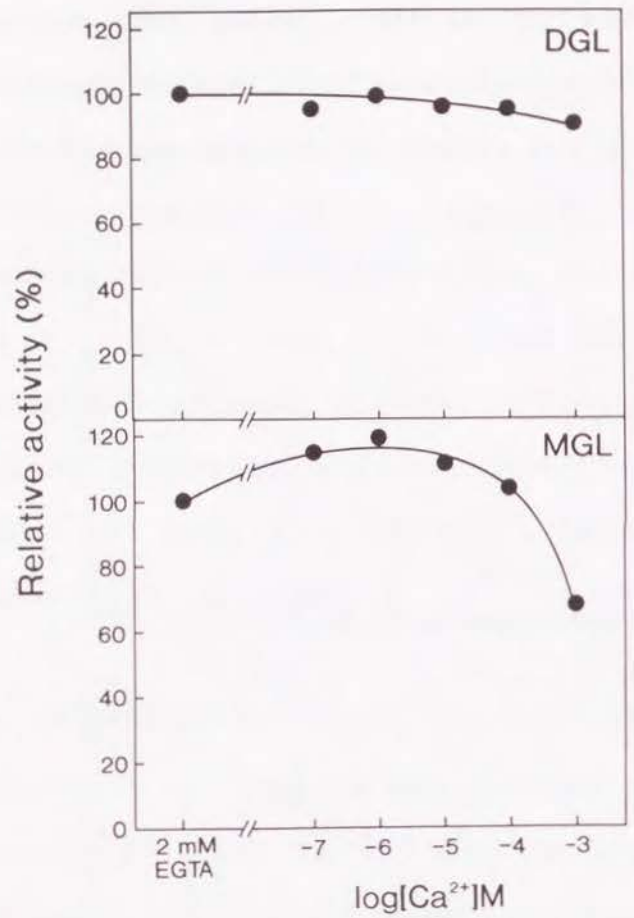

Fig. 9. Effects of Ca^{2+} on DG lipase and MG lipase activities from platelet microsome. DG lipase or MG lipase activities were measured in the buffer containing various concentrations of Ca^{2+} and 100 μM of 1-[^{14}C]stearoyldiacylglycerol or 2-[^{3}H]arachidonoyl-monoacylglycerol, respectively. [Ca^{2+}] was adjusted by Ca^{2+}-EGTA buffers, pH 7.0. The assays were performed as described in "MATERIALS AND METHODS". The results are expressed as the means for three separate experiments.

Effect of cytosolic free Ca^{2+} concentrations on arachidonic acid release from phospholipids

The Ca^{2+} ionophore, A23187 is a pharmacological tool to evoke changes in cytosolic free Ca^{2+} concentrations. The effects of A23187 on cytosolic free Ca^{2+} concentrations in the presence of extracellular Ca^{2+} were examined in fura-2-loaded human platelets. This reagent rapidly induced the concentration-dependent elevation in cytosolic free Ca^{2+} concentrations as shown in Fig. 10A. The dose-dependent curve was obtained (Fig. 10B). Thus, it was possible to control cytosolic free Ca^{2+} concentrations by varying concentrations of A23187. The cytosolic free Ca^{2+} concentration was not elevated in the presence of ONO-3708, the TXA_2 receptor antagonist, even by stimulation with collagen (Fig. 11B). Then, to study the relationship with cytosolic free Ca^{2+} concentrations and the AA release by changing the cytosolic free Ca^{2+} level by adding various concentrations of A23187 in collagen-stimulated or unstimulated platelets. The changes of cytosolic free Ca^{2+} concentrations were independent regardless of whether collagen stimulation or not (Fig. 11C,D). Only a very small amount of AA metabolites was formed

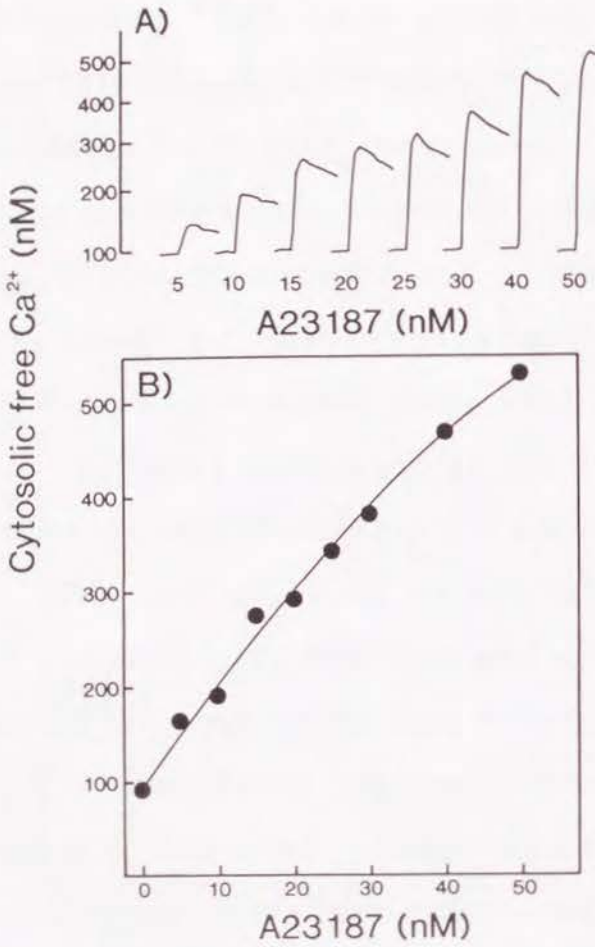

Fig. 10. Changes in cytosolic free Ca^{2+} concentrations in fura-2-loaded platelets during stimulation of human platelets with various concentrations of A23187.

A. Fluorescence signals monitored in response to various concentrations of A23187. In the presence of 5 mM $CaCl_2$, A23187 at the concentrations indicated was added to fura-2 loaded platelet suspensions (final volume : 0.4 ml) at 37°C. Cytosolic free Ca^{2+} concentrations were monitored as described in the "MATERIALS AND METHODS". The figure shows representative traces from triplicate experiments.

B. Cytosolic free Ca^{2+} levels during stimulation of human platelets with various concentrations of A23187. The changes of cytosolic free Ca^{2+} concentrations were monitored as described above. Maximal cytosolic free Ca^{2+} concentrations evoked at various concentrations of A23187 were plotted.

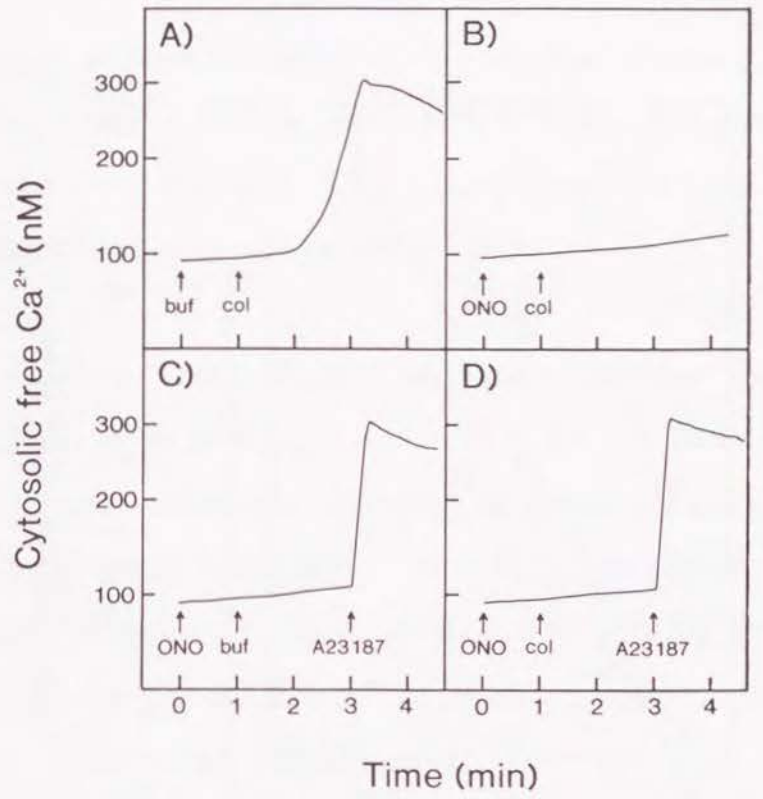

Fig. 11. The changes of cytosolic free Ca^{2+} concentrations of platelets stimulated with collagen and/or A23187 in the presence or absence of ONO-3708. Fura-2 loaded platelet suspension ($2x10^8$/ml) were stimulated by the reagents indicated and the cytosolic free Ca^{2+} concentrations were monitored as mentioned in "MATERIALS AND METHODS". ONO, ONO-3708; buf, platelet suspending buffer; col, collagen.

when collagen-unstimulated platelets were treated with 20 nM A23187 (Fig. 12). Under this condition, concentrations of cytosolic free Ca^{2+} were elevated to about 300 nM. Whereas, when collagen-stimulated platelets were treated with 20 nM A23187, concentrations of cytosolic free Ca^{2+} were similarly elevated to about 300 nM and AA metabolites were formed at the same level as in the platelets stimulated with

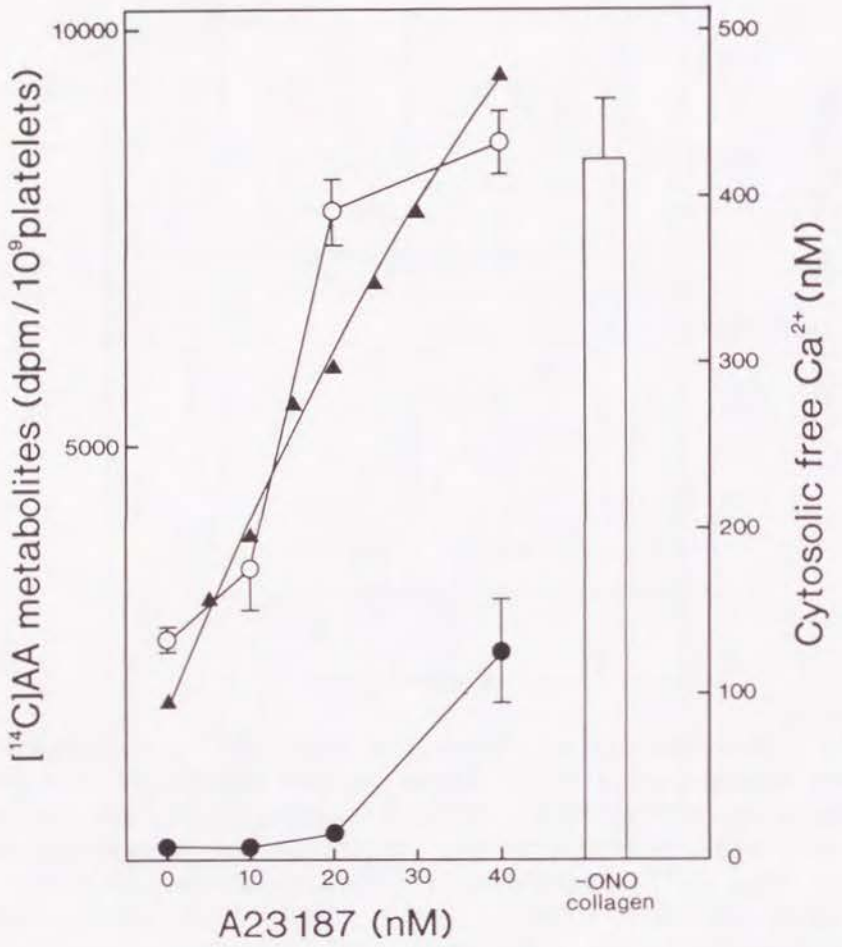

Fig. 12. A23187-induced elevation of cytosolic free Ca^{2+} concentrations and production of arachidonic acid metabolites in collagen-stimulated or unstimulated platelets in the presence of ONO-3708. In the presence of ONO-3708, [^{14}C]AA or fura-2 loaded platelet suspension was stimulated with or without collagen in the presence of various concentrations of A23187. Cytosolic free Ca^{2+} concentrations (▲) and arachidonic acid metabolites produced in collagen-stimulated (◯) or unstimulated (●) platelets were analyzed as described in "MATERIALS AND METHODS". The levels of AA metabolites produced in collagen (10 μg/ml)-stimulated platelets in the absence of ONO-3708 (open column). The data shown are means ± S.D. of triplicate determinations.

collagen without ONO-3708. In the absence of A23187, the AA metabolites were much more produced in the collagen-stimulated platelets than in the collagen-unstimulated platelets (Fig. 12).

Formation of AA metabolites and platelet responses by stimulation with STA_2

It was examined whether AA metabolites were formed by stimulation with STA_2, the TXA_2 analogue. As shown in Fig. 13A, STA_2 (up to 100 nM) could not evoke a significant formation of AA metabolites. However, the other platelet responses (cytosolic free Ca^{2+} elevation, aggregation and PLC activation) were fully induced. This induction was saturated by stimulation with 100 nM STA_2 (data not shown). The time courses of the formation of AA metabolite formation, cytosolic free Ca^{2+} elevation and platelet aggregation by stimulation with 500 nM STA_2 were examined (Fig. 13B). From the results, it may be concluded that platelets were fully activated by STA_2 without significant formation of AA metabolites.

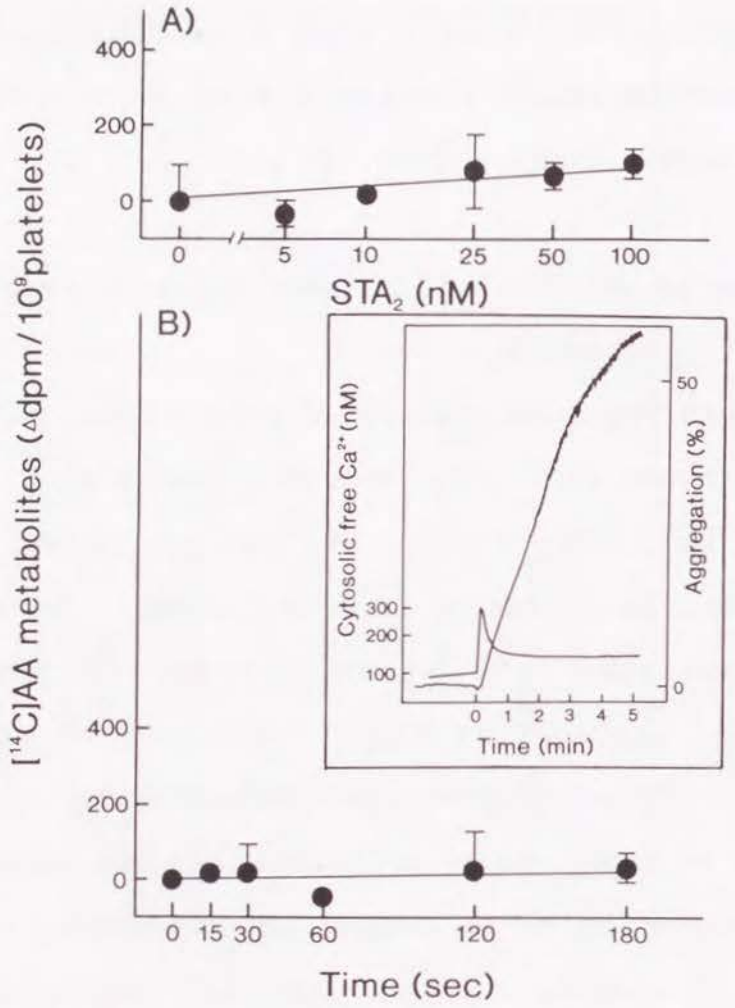

Fig. 13. STA_2 induced-AA release and platelet responses.
A. [^{14}C]AA loaded platelets were stimulated with various concentrations of STA_2 in the presence of Ca^{2+}. After stimulation for 30 sec, AA metabolites were analyzed as described in "MATERIALS AND METHODS".
B. [^{14}C]AA or fura-2 loaded platelets were stimulated with 500 nM STA_2. Cytosolic free Ca^{2+} concentrations and aggregation were continuously monitored with calcium analyzer, CAF-100. The formation of AA metabolites at the indicated time was analyzed as described in "MATERIALS AND METHODS".

DISCUSSION

Many studies have been done for revealing the mechanism of AA release in agonist-stimulated cells. In human platelets, Takamura *et al*. previously reported the mass changes of phospholipid molecular species during activation with collagen and thrombin (*9*). Thus, only phosphoinositides were hydrolyzed in collagen-activated human platelets under low cytosolic free Ca^{2+} conditions, whereas other phospholipids were degraded under high cytosolic free Ca^{2+} conditions. From these results, we can suppose three possible pathways, as shown in Fig. 14, of AA release from PI in collagen-activated human platelets under low cytosolic free Ca^{2+} concentrations. The first is the pathway catalyzed by PI-PLC, DG lipase and MG lipase, the second is by PI-PLC, DG kinase and PA-PLA_2 (*31*) and the third is by PI-PLA_2 (*32*). In this chapter, the author carried out investigations to identify the pathway(s) responsible for AA release from PI under the low Ca^{2+} concentrations which reflects early stage of activation.

One of AA metabolites, TXB_2 was formed in parallel with the formation of PA without formation of lysoPA

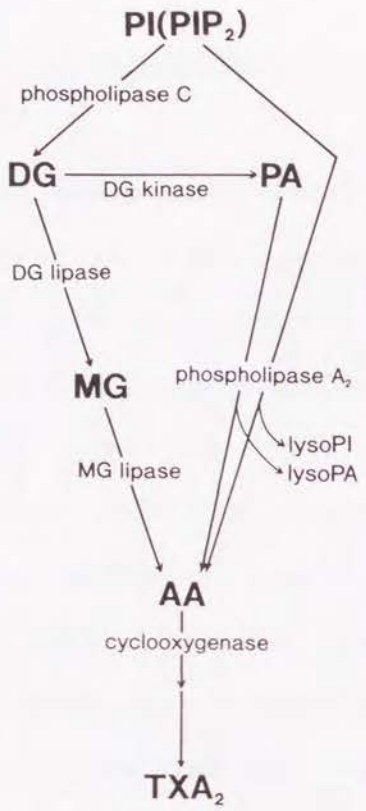

Fig. 14. The possible pathways for arachidonic acid release from phosphoinositides in human platelets.
PI, phosphoinositides; PIP_2, phosphatidylinositol 4,5-bisphosphates; DG, diacylglycerol; MG, monoacylglycerol; AA, arachidonic acid; PA, phosphatidic acid; TXA_2, thromboxane A_2.

and lysoPI in the absence of extracellular Ca^{2+}(Fig. 8). PA is the product of PI-PLC and DG kinase, whereas lyso-lipids are those of A_2 type of phospholipases (Fig. 14). Therefore, it is likely that PI-PLC may be activated under this condition, and AA may be released without activations of PLA_2. This indicates that AA may be released from phosphoinositides via PI-PLC / DG lipase / MG lipase pathway

at the early stage of stimulation of platelets with collagen, in which cytosolic free Ca^{2+} concentration was at basal level.

Then, the author tried to ascertain whether these enzymes could function under basal Ca^{2+} conditions. PI-PLC isozymes have been well known to be activated by G protein(s) or tyrosine-phosphorylation(s) under basal Ca^{2+} conditions (*33*). In fact, intact PI-PLC purified from human platelets could hydrolyze PIP_2 under nearly basal Ca^{2+} concentrations without other co-factors (see CHAPTER IV). However, the minute Ca^{2+} sensitivities of the enzymes which act at the downstream of this PI-PLC pathway was not well investigated. Hence, the author attempted to solubilize DG lipase and MG lipase activities from human platelet microsome and demonstrated that these lipases can hydrolyze the substrates under basal cytosolic free Ca^{2+} concentrations (Fig. 9). The presence of this pathway was reported in various tissues, and its physiological significance has been reported (*34-50*). Nevertheless, the molecular properties remain to be revealed.

The relationship of cytosolic free Ca^{2+} concentrations and formation of AA metabolites using Ca^{2+} ionophore, A23187, was analyzed. A23187 dose-

dependently induced cytosolic free Ca^{2+} elevation (Fig. 10). Thus, cytosolic free Ca^{2+} concentrations were able to be controled by varying concentrations of this reagent. Since AA is released to some extent by only cytosolic free Ca^{2+} elevation, it is necessary to distinguish collagen receptor dependent AA release or elevated cytosolic free Ca^{2+}-dependent AA release. Fortunately, cytosolic free Ca^{2+} elevation in collagen-activated platelets depends on TXA_2. Accordingly, elevation of cytosolic free Ca^{2+} was prevented in the presence of the TXA_2 antagonist, ONO-3708 (Fig. 11B). It was possible to control cytosolic free Ca^{2+} concentrations in collagen-stimulated or unstimulated platelets kept nearly the same level (Fig. 11 C,D). The amount corresponding to about one third of AA metabolites formed in collagen-stimulated platelets without ONO-3708 was formed in collagen-stimulated platelets in the presence of ONO-3708, in which cytosolic free Ca^{2+} concentration was at resting level (Fig. 12). This AA release was independent of Ca^{2+} elevation. Therefore, collagen may evoke AA release under basal Ca^{2+} conditions. This release may be caused via the PI-PLC / DG lipase / MG lipase pathway by consideration of above results. AA metabolites in

the collagen-unstimulated platelets were very little and not increased even by stimulation with 20 nM A23187. Under this condition, cytosolic free Ca^{2+} concentrations were elevated to about 300 nM. When cytosolic free Ca^{2+} concentration in collagen-stimulated platelets was elevated to about 300 nM by stimulation with 20 nM A23187, abandunt release of AA was observed. These results suggest that elevation of cytosolic free Ca^{2+} to about 300 nM without collagen stimulation was insufficient for full AA release. Both collagen binding and cytosolic free Ca^{2+} elevation may be necessary for full AA release. Since AA was released from PC, PE and PI by PLA_2 under high Ca^{2+} conditions (9), full PLA_2 activation may be performed synergistically by both Ca^{2+} elevation and collagen-binding. The molecular mechanism of the stimulatory effect of collagen binding for PLA_2 activation is not clear. Several factors have been proposed to be responsible for the PLA_2 activation. Pollock *et al.* (7) demonstrated that protein kinase C was not involved in the stimulatory effect of collagen for AA release.

In conclusion, the author has shown that the mechanism of AA release during activation with collagen is composed by three steps as follows (Fig. 15).

First, at the early stage of stimulation, a small amount of AA is released from PI via PI-PLC / DG lipase / MG lipase pathway, which can be activated under basal Ca^{2+} conditions. By the subsequent conversion to TXA_2, a small amount of TXA_2 at nM level can elevate cytosolic free Ca^{2+} concentrations (CHAPTER I, II). Then, PLA_2 is activated by synergistic action of elevated Ca^{2+} level and collagen binding. The activated PLA_2 hydrolyzes a large amount of AA from major phospholipids, which is converted to TXA_2. Finally, a large amount of TXA_2 activates either collagen-stimulated platelets or resting platelets. However, the platelets stimulated with TXA_2 alone are aggregated without producing further AA. This may be the termination process of the cascade responses against collagen stimulation.

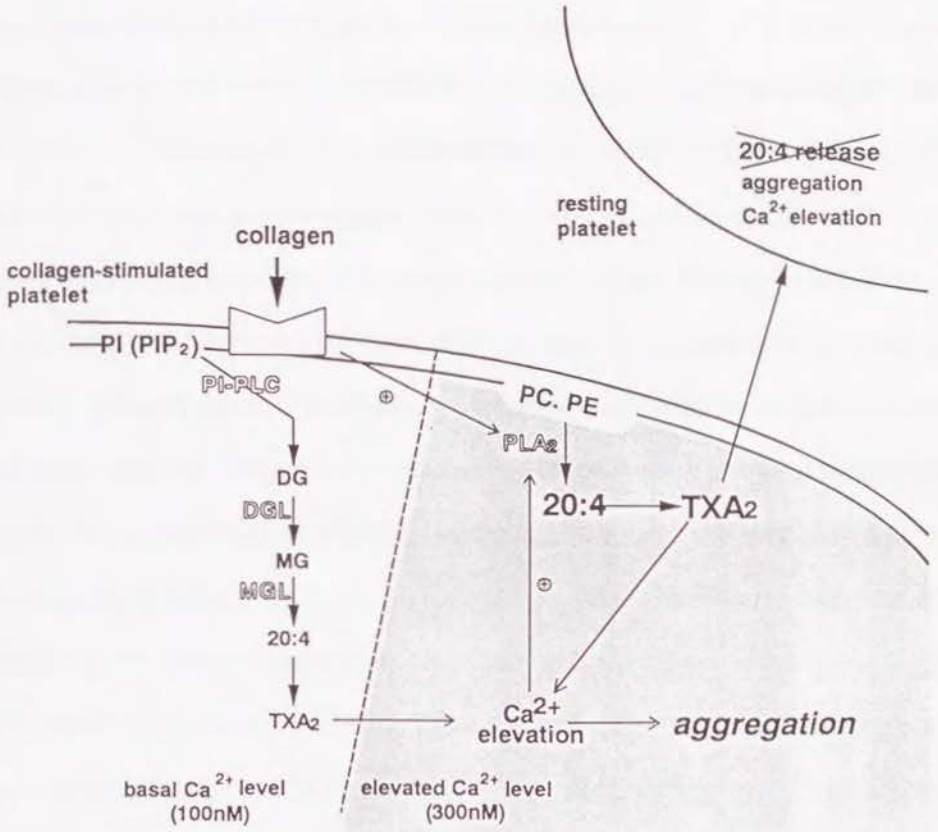

Fig. 15. The postulated scheme for arachidonic acid cascade and signal transduction in human platelets during stimulation with collagen.
See "DISCUSSION" for explanation. DGL, DG lipase; MGL, MG lipase; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

CHAPTER IV

PURIFICATION OF POLYMERIC PHOSPHOLIPASE Cs FROM HUMAN PLATELETS

When cells are stimulated with hormones, neurotransmitters, or growth factors, the metabolism of phosphoinositides is accelerated (*18,51*) and second messengers are produced. The initial reaction in a cell in response to such stimulation is the hydrolysis of phosphoinositides by PI-specific phospholipase C (PLC). One product of this reaction, inositol 1,4,5-trisphosphate (IP_3), mobilizes Ca^{2+} (*26*). The other product, diacylglycerol (DG), activates protein kinase C (*52*) and is a source of arachidonate (*9,29*), which is converted into eicosanoids to amplify the initial signal. Many studies have been carried out to elucidate the mechanisms by which PLC is activated in the initial step of the signal transduction. It is known that some tissues and cells contain distinct molecular species of PLC (*53-60*). In human platelets, Low *et al.* (*61,62*) reported the appearance of four activity peaks (420, 280, 140, and 100 kDa) for PLC on gel-filtration analysis. Manne and Kung (*63*) isolated

a PLC of 98 kDa from the cytosol of human platelets. The purified enzyme was separated into 58, 45, and 38 kDa fragments on SDS-PAGE. Banno *et al.* (*64*) also partially purified three forms of PLC (120, 67, and 70 kDa) from the same source. Baldassare *et al.* (*65*) purified a PLC of 57 kDa from the cytosol; it was immunologically distinct from the membrane-associated PLCs. However, it is unknown whether or not such multiplicity is due to native polymorphic enzymes, since it cannot be excluded that the heterogeneity of PLC in the cytosol of human platelets is due to proteolytic degradation of the enzyme.

In order to determine the real nature of PLC in human platelets, the author attempted to purify intact PLC from the cytosolic fraction of human platelets under conditions such that proteinase activities are inhibited, and to determine the immunological properties of the purified PLCs.

MATERIALS AND METHODS

Materials——PI (soybean) and PIP_2 (bovine brain) were purchased from Sigma (St. Louis, MO, U.S.A.). Aquasol-2, [^{3}H]PI (5.5 Ci/mmol), and [^{3}H]PIP_2 (2.8

Ci/mmol) were from Du Pont-New England Nuclear (Boston, MA, U.S.A.). Leupeptin was from the Peptide Institute (Osaka, Japan). PMSF and dithiothreitol were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). TSK gel butyl-Toyopearl, TSK gel heparin-Toyopearl, TSK gel DEAE-Toyopearl Pak 650M, and TSK gel G3000SW columns were from Tosoh (Tokyo, Japan). The silver staining kit was from Daiichi Pure Chemicals, Ltd. (Tokyo, Japan). The biotin blotting kit, Zeta-Probe blotting membrane, and Mini trans-blot cell were from Bio-Rad laboratories. All other chemicals were of reagent grade and obtained from commercial sources.

Purification Procedure—— Outdated human platelets were used within four days after drawing. A platelet suspension in the washing buffer [Tris / citrate / bicarbonate buffer containing 2 mM EDTA (pH 6.5)] (*10*) was centrifuged at 100 x *g* for 30 min to remove red cells. The supernatant was centrifuged at 1500 x *g* for 30 min, and the resultant pellet was washed once in the lysis buffer [20 mM Tris/HCl (pH 7.4), 20 mM EGTA, 0.5 mM leupeptin, 0.1 mM dithiothreitol], followed by sonication twice with a Branson sonicator model 250 at setting 3 for 30 sec. The sonicated platelets were

centrifuged at 200,000 x *g* for 1 h and thus separated from the membrane fraction. The supernatant was diluted with buffer A [20 mM Tris/HCl(pH 7.4), 5 mM EDTA, 0.5 mM PMSF, 0.1 mM dithiothreitol], and A_{280} was adjusted to 20. Solid $(NH_4)_2SO_4$ was added to 0.18 saturation, followed by stirring for 20 min in an ice bath. The resultant solution was centrifuged at 10,000 x *g* for 20 min to remove the precipitate. Solid $(NH_4)_2SO_4$ was further added to the supernatant to 0.38 saturation and the resultant precipitate was stored at 4^oC.

The $(NH_4)_2SO_4$ precipitate from approximately 300 units of platelets was dialyzed against buffer A and the resultant dialysate was diluted with an equal volume of buffer A. The diluted protein solution was centrifuged at 200,000 x *g* for 30 min to remove the insoluble precipitate. The supernatant was loaded on a DEAE-Toyopearl pak 650M column (2.2 cm x 20 cm) that had been equilibrated with buffer A. The column was washed with 2 bed volumes of buffer A, followed by 600 ml of buffer A containing a linear NaCl gradient (0 - 0.4 M). The major activity was eluted at 0.2 M NaCl and the corresponding fractions were pooled.

The active fractions from DEAE-Toyopearl columns

were dialyzed against buffer B [20 mM Tris/HCl (pH 7.4), 5 mM EDTA, 0.5 mM PMSF, 0.1 mM dithiothreitol, 0.8 M $(NH_4)_2SO_4$] and the resultant dialysate was applied to a butyl-Toyopearl column (2.8 cm x 32 cm). After washing with 1.5 bed volumes of buffer B, proteins were eluted with a linear gradient of from 100% buffer B to 100% buffer A. Two activity peaks were detected. The first peak material was named PLC-I and the other one PLC-II. The fractions corresponding to PLC-I and PLC-II were pooled separately. The above processes, involving approximately 300 units of platelets, were repeated four times.

The pooled PLC-I and PLC-II fractions were separately dialyzed against buffer C [20 mM Tris/HCl (pH 7.4), 5 mM EDTA, 0.5 mM PMSF, 0.1 mM dithiothreitol, 0.1 M NaCl] and then applied to heparin-Toyopearl columns (1.0 cm x 20 cm) equilibrated with the same buffer. Activity peaks were eluted with linear 0.1 M - 0.7 M NaCl gradients in the same buffer. The solutions containing PI-hydrolysis activity were precipitated by 0.75 saturation with $(NH_4)_2SO_4$.

The precipitates from heparin-Toyopearl columns were dissolved in 300 µl of buffer C, and the resultant

solutions were centrifuged at 135,000 x *g* for 30 min. The resultant supernatants were applied to G3000SW columns (7.5 mm x 60 cm) equilibrated with buffer C. The flow rate was 0.4 ml/min. A single activity peak was eluted in each case. The fractions containing the activity were pooled and stored at -20°C.

The pooled active fractions from G3000SW columns were rechromatographed on the same columns. A single protein peak was eluted which coincided with the activity in each case. The active fractions were collected, frozen with liquid N_2, and then stored at -80°C.

Assay for PLC Activity——PLC activity was assayed by measuring the formation of radioactive inositol monophosphate from [^{3}H]PI, as follows. The reaction mixture (100 µl) comprised 50 µl of the assay mixture [80 mM PIPES/HCl (pH 7.0), 6 mM $CaCl_2$], 10 µl of the substrate mixture, and 40 µl of the elution buffer containing the enzyme. The substrate mixture, comprising 0.4µM [^{3}H]PI and 0.5 mM PI (soybean) in 10 mg/ml sodium deoxycholate, was prepared by vortexing and sonication twice with a Branson sonicator model 250 at setting 8 for 1 min. After incubation for 3-30 min

at 37°C, the reaction was terminated by the addition of 0.5 ml of chloroform / methanol / concentrated HCl (100 : 100 : 0.6, v/v), and 0.15 ml of 5 mM EGTA, 1 N HCl. The mixture was shaken vigorously with a vortex-mixer and then centrifuged at 1000 x *g* for 10 min. A 0.38 ml portion of the upper aqueous phase was transferred to a vial containing 4 ml of Aquasol-2, and then the radioactivity was determined with a liquid scintillation counter (Packard, TRI-CARB 4530). Certain variations of this standard assay are indicated in the figure legends.

Other Methods——SDS-PAGE was carried out by the method of Laemmli (*66*) in 7.5% polyacrylamide slab gels. A silver-staining kit was used to visualize protein bands. Protein was assayed with the Bio-Rad protein assay kit, with γ-globulin as the standard. Electrophoretic transfer of protein from slab gels to a Zeta-Probe membrane was performed using a Mini trans-blot cell (Bio-Rad). It was confirmed by using a Biotin-Blotting Kit (Bio-Rad) that the proteins were blotted to the membrane. Immunoblotting using specific antibodies against four rat PLC isozymes (PLC-β, γ_1, γ_2, δ) was carried out as described in (*67*).

RESULTS

Purification of PLCs——When cytosolic proteins of human platelets were fractionated with $(NH_4)_2SO_4$, about 80% of the total PLC activity was concentrated between 0.18 and 0.38 saturation of $(NH_4)_2SO_4$. The PLC activity was separated into two peaks on a DEAE-Toyopearl column. The major activity was eluted at 0.3 M NaCl and the minor one at 0.15 M NaCl (Fig. 16A).

In the experiment with platelets incubated for 10 days after drawing blood, the latter peak was found to have increased (data not shown), suggesting that the minor activity peak was due to secondary metabolic products of PLC. Hence, the major active peak material was subjected to further purification. The fractions corresponding to the major activity peak were collected, precipitated with $(NH_4)_2SO_4$, and then dialyzed against buffer B (see "MATERIALS AND METHODS"). The dialysate was centrifuged to remove the insoluble precipitate. The supernatant was applied to a butyl-Toyopearl column. Two activity peaks were eluted. The first peak (termed PLC-I) was eluted at 0.46 M $(NH_4)_2SO_4$ and the second one (termed PLC-II) at 0.25 M (Fig. 16B). The fractions

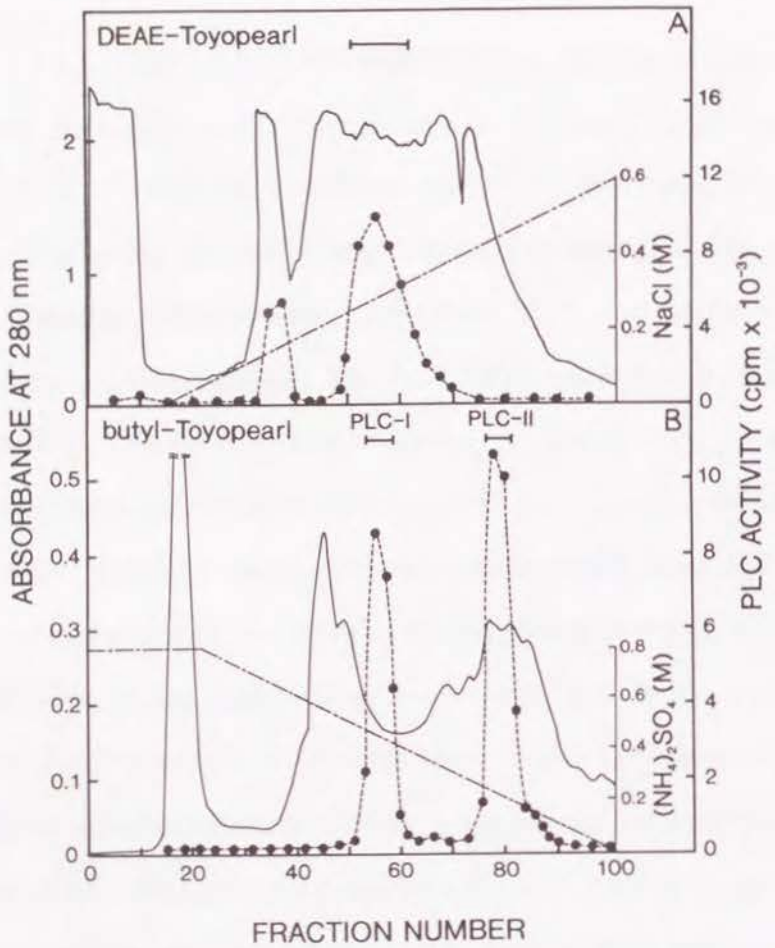

Fig. 16. DEAE-Toyopearl pak 650M HPLC column chromatography of the human platelet cytosolic fraction (A); butyl-Toyopearl column chromatography of PLC obtained from the DEAE-Toyopearl Pak 650M column chromatography (B). A, The diluted human platelet cytosolic fraction was applied to a DEAE-Toyopearl pak 650M HPLC column and eluted as described under "MATERIALS AND METHODS". PLC activities were measured with [^{3}H]PI as the substrate (--●--). The active fractions, indicated by a bar, were pooled. —·—, NaCl concentration; ———, absorbance at 280 nm.
B, The PLC solution eluted from the column in A was applied to a butyl-Toyopearl column and eluted as described under "MATERIALS AND METHODS". PI-hydrolyzing activities were measured (--●--), and the active fractions corresponding to PLC-I and PLC-II were pooled separately. —·—, $(NH_4)_2SO_4$ concentration; ———, absorbance at 280 nm. The values in both A and B were all obtained in one experiment; three other experiments gave similar results.

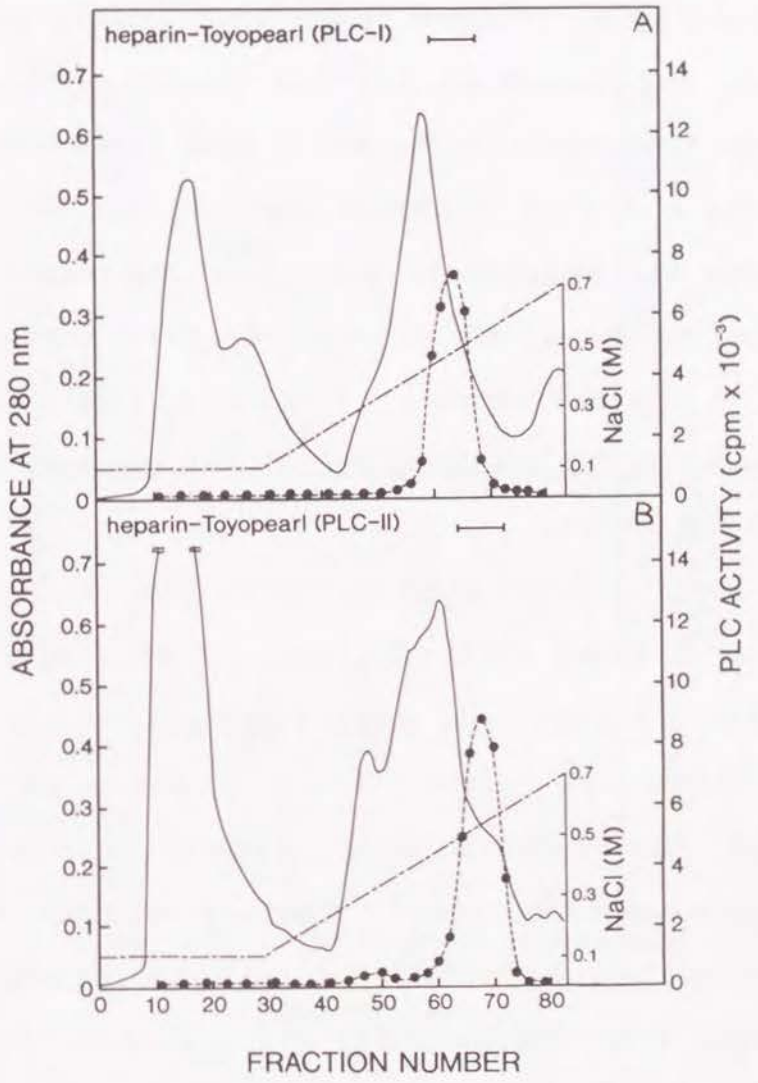

Fig. 17. Heparin-Toyopearl column chromatography of PLC-I (A) and PLC-II (B). PLC-I (A) and PLC-II (B) obtained from the butyl-Toyopearl column were applied to heparin-Toyopearl columns and eluted as described under "MATERIALS AND METHODS".
--●--, PLC activity for PI-hydrolysis; ———, absorbance at 280 nm; —·—, NaCl concentration.

corresponding to the two peaks were pooled separately and then subjected to further purification. The fractions corresponding to PLC-I were precipitated with $(NH_4)_2SO_4$ and then dialyzed against buffer C. The dialysate was applied to a heparin-Toyopearl column. The major protein, which was free from activity, was eluted in the flow-through fractions. A single peak of PI-hydrolyzing activity was eluted between 0.43-0.53 M NaCl (Fig. 17A).

The fractions corresponding to the activity peak were precipitated with $(NH_4)_2SO_4$, dissolved in 300 µl of buffer C, and then subjected to G3000SW column chromatography. Four major protein peaks were obtained, the PI-hydrolyzing activity coinciding with the second one (Fig. 18A). The active fractions were rechromatographed, a single protein peak coinciding with the activity being eluted (Fig. 18B). The apparent molecular mass of PLC-I was estimated to be 440 kDa from a calibration curve (Fig. 20). PLC-II was purified by the same procedure. The elution position of PLC-II from a heparin-Toyopearl column was almost the same as that in the case of PLC-I (0.49-0.58 M NaCl). PLC-II was eluted as a single activity peak together with a single protein peak on rechromatography

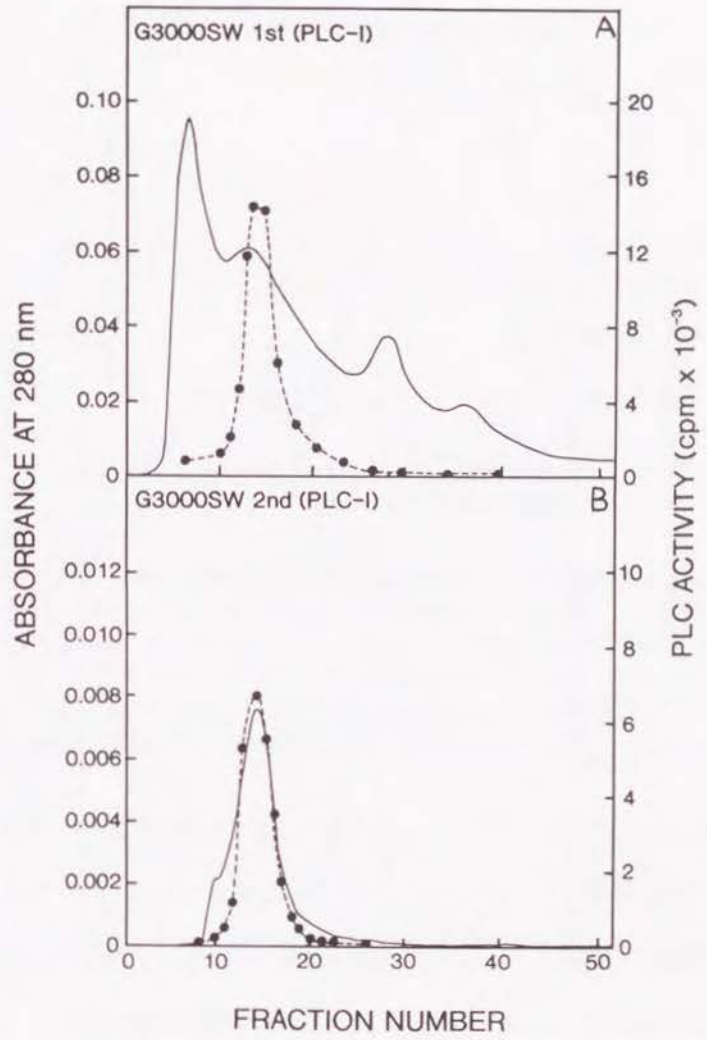

Fig. 18. First (A) and second G3000SW HPLC column chromatography (B) of PLC-I. A, PLC-I obtained from the heparin-Toyopearl column was applied to a G3000SW HPLC column. The eluted fractions were assayed for PLC activity for PI hydrolysis as described under "MATERIALS AND METHODS". --●--, PLC activity for PI hydrolysis; ———, absorbance at 280 nm. B, The active fractions from the first G3000SW column were again applied to the same column. The eluted fractions were assayed for assaying for PI-hydrolyzing activity as described under "MATERIALS AND METHODS." --●--, PLC activity for PI hydrolysis; ———, absorbance at 280 nm.

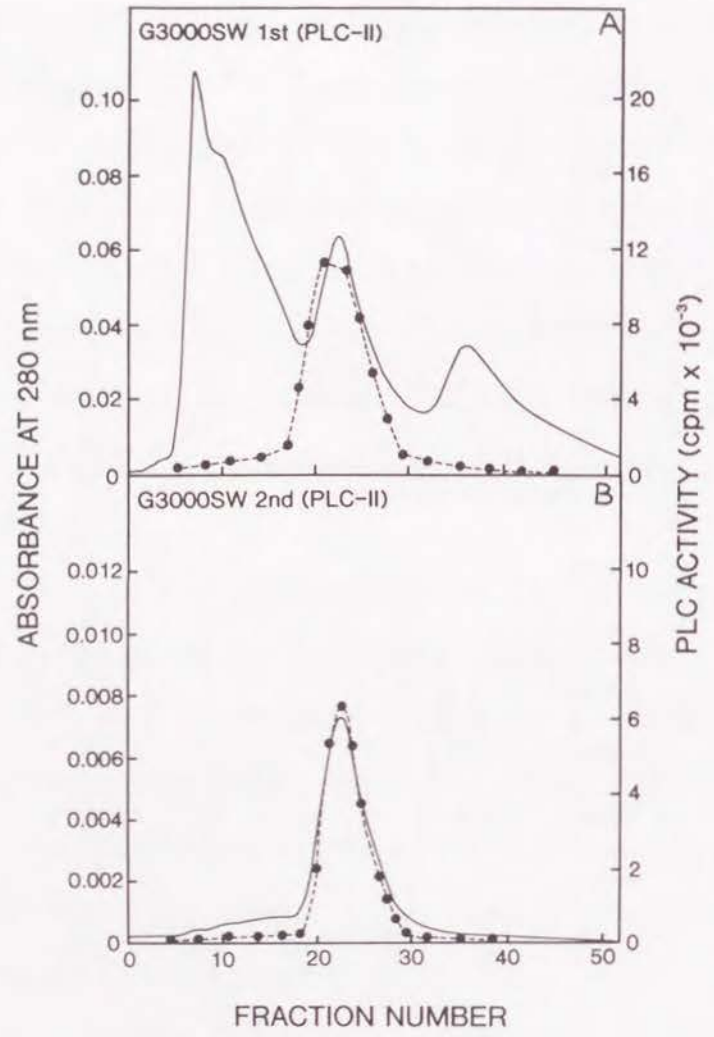

Fig. 19. First (A) and second G3000SW HPLC column chromatography (B) of PLC-II. A, PLC-II obtained from the heparin-Toyopearl column was applied to a G3000SW HPLC column. The eluted fractions were assayed for PLC activity as described under "MATERIALS AND METHODS". ---●---, PLC activity for PI hydrolysis; ———, absorbance at 280 nm.
B, The active fractions from the first G3000SW HPLC column were again applied to the same column. The eluted fractions were assayed for PI-hydrolyzing activity as described under "MATERIALS AND METHODS". ---●---, PLC activity for PI hydrolysis; ———, absorbance at 280 nm.

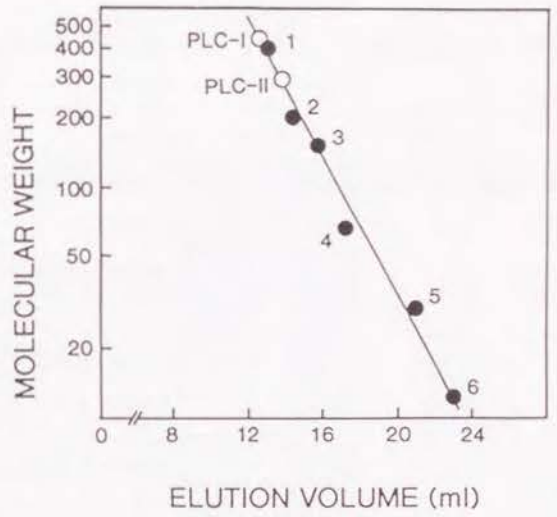

Fig. 20. Molecular weight estimation of the two purified forms of PLC by gel filtration on a G3000SW column. Chromatography was carried out as in Figs. 3 and 4. The markers used were: 1, ferritin (400 kDa); 2, β-amylase (200 kDa); 3, alcohol dehydrogenase (150 kDa); 4, bovine serum albumin (66 kDa); 5, carbonic anhydrolase (29 kDa); 6, cytochrome c (12,4 kDa).

on a G3000SW column (Fig. 19). The apparent molecular mass of PLC-II was estimated to be about 290 kDa from the calibration curve (Fig. 20). The two purified PLCs were analyzed by SDS-PAGE, and a single band was visualized on silver-staining in each case at the same position from the origin (Fig. 21). The apparent mass was estimated to be 146 kDa. A summary of the purification is presented in Table 1. The specific activities increased 1348fold (PLC-I) and 1441 fold (PLC-II) as compared with the starting cytosolic activity, though the yields were low (PLC-I, 0.38%; PLC-II, 0.33%).

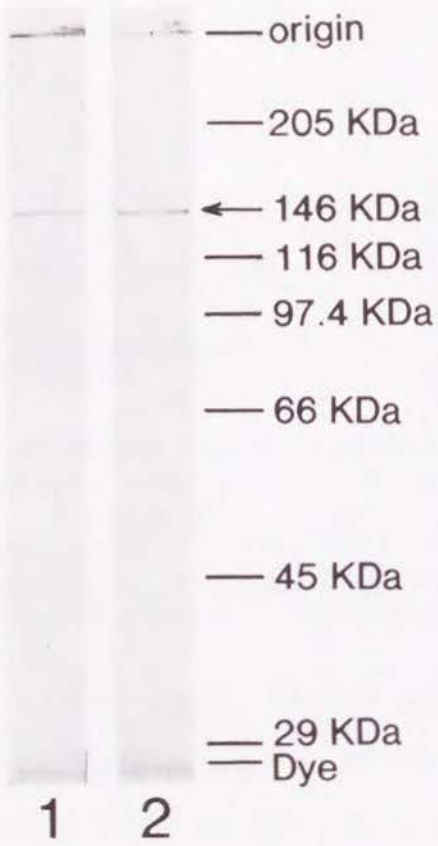

Fig. 21. SDS-PAGE of PLC-I and PLC-II. The PLC-I and PLC-II obtained by rechromatography on the G3000SW column were applied to lanes 1 and 2, respectively. Protein bands were visualized by silver-staining. Bars indicate the mass standards (205 kDa, myosin; 116 kDa, β-galactosidase; 97.4 kDa, phosphorylase b; 66 kDa, bovine serum albumin; 45 kDa, ovalbumin; 29 kDa, carbonic anhydrolase).

Table 1

Purification of PLC-I and PLC-II from Human Platelet Cytosol

Purification step	Total protein	Total activity	Specific activity	Yield	-Fold purification
	mg	nmol/min	nmol/min/mg	%	
200,000 x g supernatant	34332.5	45553.5	1.3	100.0	1
$(NH_4)_2SO_4$ precipitation	17551.0	35792.0	2.0	78.6	1.5
DEAE-Toyopearl	920.5	16072.9	17.5	35.3	13.2
Butyl-Toyopearl					
PLC-I	41.7	2418.2	58.0	5.31	43.6
PLC-II	82.6	3885.8	47.0	8.53	35.3
Heparin-Toyopearl					
PLC-I	8.75	2336.9	267.1	5.13	200.8
PLC-II	7.02	2016.3	287.2	4.43	215.9
G3000SW (1st)					
PLC-I	0.77	1285.3	1669.2	2.82	1255.0
PLC-II	0.62	1119.0	1804.8	2.46	1357.0
G3000SW (2nd)					
PLC-I	0.097	173.9	1792.8	0.38	1348.0
PLC-II	0.079	151.4	1916.5	0.33	1441.0

Immunological Properties of the Purified PLCs---When PLC-I and II were analyzed for their immunoreactivities with antibodies to rat PLC-β, γ_1, γ_2, and δ, similar results were obtained with PLC-I and II. They reacted with anti-PLC-β and δ weakly but did not react with anti-PLC-γ_1 and γ_2 (data not shown).

Substrate Specificity, pH optimum, and Ca^{2+}-Sensitivity of the Purified PLCs---The PI- and PIP_2-hydrolyzing activities of the two purified enzymes were determined at various pHs and Ca^{2+} concentrations. As shown in Figure 22A, PLC-I and II had pH optima for

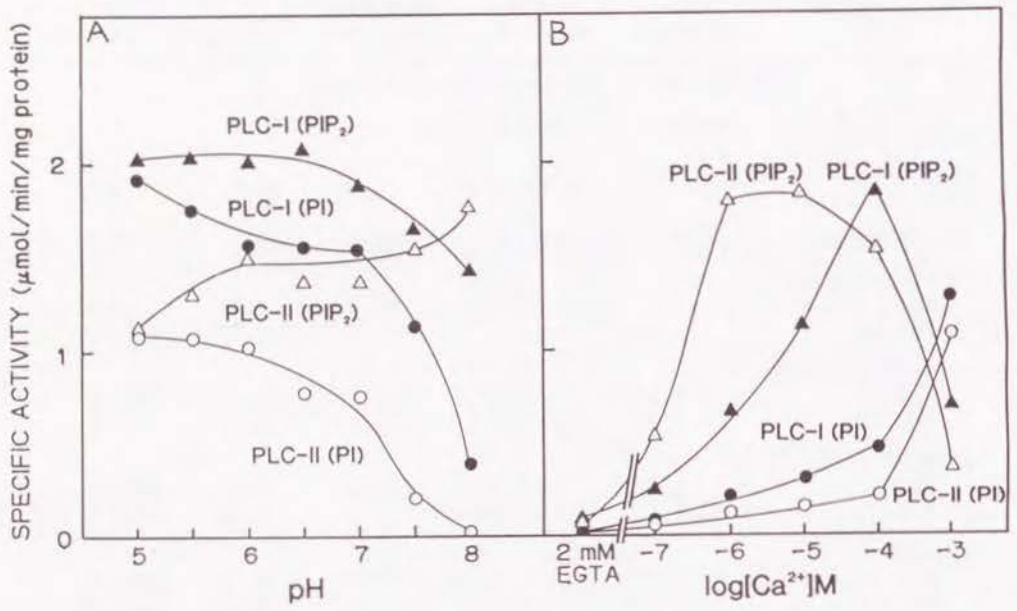

Fig. 22. pH and calcium dependence of PLC-I and PLC-II in PI- and PIP_2-hydrolysis. PLC-I (solid symbols) or PLC-II (open symbols) activity was measured in a reaction mixture containing PI (●, ○) or PIP_2 (▲, △) as a substrate. A, pH dependence of PLC-I and PLC-II. The reaction mixture (100 μl) contained 20 mM Tris/maleate buffer (pH 5.0-8.0), 0.1 mM PI or PIP_2, [^{3}H]PI (70,000 dpm) or [^{3}H]PIP_2 (20,000 dpm), and 0.04 mM PE. The substrate was prepared as small unilamellar vesicles by sonication. The calcium concentrations used for PI- and PIP_2-hydrolysis were adjusted to 1 mM and 100 μM with Ca^{2+}-EGTA buffers (*30*), respectively. B, Calcium dependence of PLC-I and PLC-II. Various free Ca^{2+} concentrations were obtained with Ca^{2+}-EGTA buffers (pH 7.0) (*30*). The reaction mixture (100 μl) contained 40 mM PIPES/HCl buffer (pH 7.0), 0.1 mM PI or PIP_2, [^{3}H]PI (70,000 dpm) or [^{3}H]PIP_2 (20,000 dpm), and 0.04 mM PE. The substrate was prepared as described in (A). The results are expressed as the means for three separate experiments.

PIP_2 hydrolysis from pH 5 to 7 and from 6 to 8, respectively, and for PI hydrolysis from acidic pH to 7.0. This indicates that both PLC-I and II may function under physiological pH conditions. The PI-hydrolyzing activities were maximal at 1 mM Ca^{2+} (Fig. 22B). On the other hand, the PIP_2-hydrolyzing activities were maximal at 100 μM (PLC-I) and 1-10 μM (PLC-II) Ca^{2+}. However, the hydrolytic activities toward PIP_2 were remarkably decreased at 1 mM Ca^{2+}.

DISCUSSION

Many tissues and cells contain multiple forms of PLC with different molecular masses (*68*). Ryu *et al.* (*57-59*) purified three PLCs from bovine brain and showed them to be immunologically distinct from each other. Homma *et al.* (*60*) purified a new type of PLC, which was more specific for PIP_2, from rat brain and proved it to be an immunologically new isozyme. Thus, the brain contains at least four immunologically distinct PLC isozymes. In addition, Hofmann and Majerus (*53*) purified two immunologically distinct PLCs (65 and 85 kDa) from sheep seminal vesicles, and Nakanishi *et al.* (*55*) separated two different PLCs (71

and 140 kDa) from rat liver, suggesting that tissues other than brain also contain multiple PLC isozymes with different masses.

In human platelets, Low *et al.* (*61,62*) observed four activity peaks on gel-filtration column chromatography. Banno *et al.* (*64*) partially purified three forms of PLC (120, 70, and 67 kDa) from the cytosol of human platelets, and Manne and Kung (*63*) purified a 98 kDa PLC from the same source and observed three enzymes (58, 45, and 38 kDa) on SDS-PAGE. Baldassare *et al.* (*65*) purified a 57 kDa PLC to homogeneity from the cytosolic fraction. Thus, multiple forms of PLC are present in human platelets. However, it is uncertain whether these PLCs are intact or modified forms. The author found that incubating platelet cytosolic fraction in the presence of Ca^{2+} resulted in conversion of high mass forms of PLC (250-440 kDa) into lower mass forms (70-100 kDa)(data not shown). This suggested that the conversion was due to a Ca^{2+}-dependent proteinase. In fact, Low *et al.*(*62*) showed that partially purified high mass forms of PLC were modified by the partially purified proteinase into lower mass forms. Thus, it seems likely that Ca^{2+}-dependent proteinases were responsible for the

modification of multiple forms of PLC in human platelets. The author therefore attempted to purify intact PLC under conditions such that the modification of enzymes by Ca^{2+}-dependent proteinases would be prevented. The author lysed platelets in the presence of 20 mM EGTA and 0.5 mM leupeptin, and carried out all purification procedures in the presence of 5 mM EDTA and 0.5 mM PMSF. Two high mass forms of PLC (440 and 290 kDa) were obtained. Both purified PLCs were found to be composed of a monomer of 146 kDa on SDS-PAGE (Fig. 21). Thus, PLC-I and PLC-II were determined to be a trimer and a dimer of the 146 kDa polypeptide, respectively, the occurrence of which was previously suggested by Low *et al.* (*62*). PLC-I has the largest mass among the forms which have been purified from human platelets (*63-65*). This suggests that PLC-I and PLC-II are intact PLCs which were protected from proteolytic modification. The molecular mass of the monomeric form of PLC is similar to those of PLCs from rat liver, bovine brain, and bovine platelets (*53,55-57,69*).

Banno *et al.* (*64*) reported that no changes were observed in the elution profiles of the three enzymes on gel filtration, regardless of whether or not

proteinase inhibitors were present during the purification processes, suggesting that the multiple forms may not be the result of proteolysis. The present results hardly support that proposal.

Baldassare *et al.* (*65*) detected two activity peaks with masses of 57 and 200 kDa in the cytosolic fraction. The antibody of the 57 kDa enzyme inhibited the activity of the 200 kDa enzyme. Hence, the former was suggested to be derived from the latter. It is possible that the 200 kDa form was processed from the 290 kDa form described here.

None of the antibodies for the bovine brain PLCs is reported to recognize the platelet PLC of 140 kDa in immunoblot experiments (57). In this experiment, the antibodies to rat PLCs-β and δ cross similarly with the 146 kDa monomers from the 290 and the 440 kDa PLCs, though the crossings were weak. Hence it seems likely the monomers of the 290 and 440 kDa enzymes are homologous. However, it is difficult to explain the dual responses of the 146 kDa monomer to the two antibodies. It cannot be excluded that this was caused by the unique reactivity of human PLC to rat antibodies. The antibody to PLC-δ reacted most intensively, so these enzymes appear to be PI-PLC with

X-Y putative catalytic site (*70*), since the epitopes of the antibody are present in the site (*67*). This suggests that the polymeric PLCs from human platelets are immunologically novel isozyme(s) among the 140-150 kDa PLC isozymes (Fig. 23).

During preparation of this paper, a cytosolic(c) PLC of 145 kDa was isolated from human platelets (*71*) which crossed with rat PLC-γ_2, though PLC-I and II did not. In addition, the pH optima of both PLC-I and PLC-II for PIP_2 hydrolysis were around neutral pH, whereas that of cPLC was at acidic pH (5.0-5.5). PLC-II effectively hydrolyzed PIP_2 at the Ca^{2+} concentration below 1 μM. These results suggest that the functions of PLC-I and II are different from that of cPLC (Table 2).

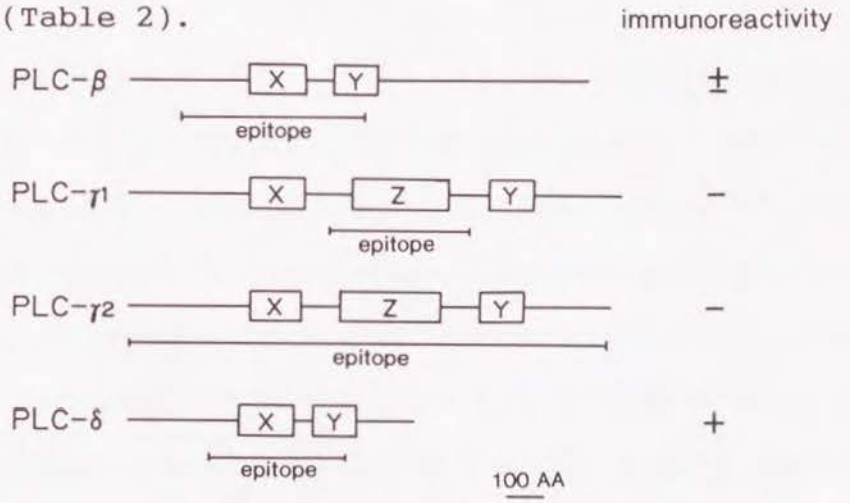

Fig. 23 Molecular structures of PLC isozymes and immunoreactivities of PLC-I and II with the antibodies for each isozymes. See "DISCUSSION" for explanation.

Table 2
PROPERTIES OF CYTOSOLIC PLCs FROM HUMAN PLATELETS

		cPLC-I	PLC-I	PLC-II
Mr (kDa)		145	440 (3x146)	290 (2x146)
Optimal $[Ca^{2+}]$	(PI)	0.1-1mM	1mM	1mM
	(PIP_2)	1μM	100μM	1-10μM
Optimal pH	(PI)	5.5-6.0	5.0	5.0
	(PIP_2)	5.0	5.0-7.0	6.0-8.0
Immunological Property		γ_2	$\beta(\pm)$, δ	$\beta(\pm)$, δ

SUMMARY

CHAPTER I

The thromboxane A_2 antagonist, ONO-3708, completely inhibited the increase in cytosolic free Ca^{2+} in human platelets during activation with collagen. Half-maximal Ca^{2+} release and influx required about 3 and 4 nM STA_2, a stable thromboxane A_2 mimetic, respectively. However, half maximal activation of phospholipase C required about 18 nM STA_2. This suggests that thromboxane A_2 directly causes Ca^{2+} mobilization without further activation of phospholipase C during activation of human platelets with collagen.

CHAPTER II

The Ca^{2+} channel blocker, nifedipine, a dihydropyridine derivative, inhibited the Ca^{2+} influx and release from internal stores caused by collagen or a low concentration of the TXA_2 analogue, STA_2 (10 nM), but did not inhibit those caused by thrombin or a high concentration of STA_2 (100 nM). These results indicate the presence of two distinct, dihydropyridine-sensitive and insensitive, Ca^{2+} channels dependent on

the concentrations and classes of agonists in human platelets.

CHAPTER III

The mechanism of arachidonic acid release in collagen-activated human platelets was studied. One of arachidonic acid metabolites, thromboxane B_2 (TXB_2) was formed in parallel with the formation of phosphatidic acid (PA) without formation of lyso-phosphatidic acid (lysoPA) and lysophosphatidylinositol (lysoPI) in the absence of extracellular Ca^{2+}, suggesting that AA was released from PI via PI-specific phospholipase C (PI-PLC) / diacylglycerol (DG) lipase / monoacylglycerol (MG) lipase pathway under the cytosolic low Ca^{2+} concentrations. Moreover, solubilized DG lipase and MG lipase could hydrolyze the substrates at basal cytosolic free Ca^{2+} concentrations. Subsequently, the relationship of cytosolic free Ca^{2+} concentrations and formation of AA metabolites was analyzed using Ca^{2+} ionophore, A23187. Collagen was able to induce a release of small amounts of AA under basal cytosolic Ca^{2+} conditions. However, a release of high amounts of AA was induced by phospholipase A_2 activated by both collagen-receptor occupancy and

elevated Ca^{2+} levels. The TXA_2 mimetic agonist, STA_2 induced all the responses except for AA release. From these results, the mechanism of AA release and signal transduction in collagen-activated human platelets was discussed.

CHAPTER IV

Two types of cytosolic phospholipase C specific for phosphoinositides were purified from human platelets. The molecular masses of the purified enzymes were 440 and 290 kDa. These enzymes were concluded to be respectively a trimer and a dimer of homologous 146 kDa polypeptides. The 146 kDa polypeptide may be an immunologically novel isozyme among the 140-150 kDa PLC isozymes. Both enzymes hydrolyzed phosphatidylinositol and phosphatidyl-inositol 4,5-bisphosphate in a Ca^{2+}-dependent manner.

ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude to Dr. Makoto Kito, Professor of the Research Institute for Food Science, Kyoto University, for his kind guidance, invaluable discussions and continuous encouragement throughout the course of this study.

The author is deeply grateful to Dr. Shigeru Utsumi, Associate Professor of the Research Institute for Food Science, Kyoto University, for his kind support and useful discussions.

The author is greatly indebted to Dr. Hiroshi Narita, Associate Professor of Kyoto Women's University and Dr. Hitoshi Takamura, Lecturer of Nara Women's University, for their helpful advice, continuous support and encouragement.

The author also wishes to express his special thanks to Dr. Reiko Urade and Dr. Shin-ichi Fukuoka at the Research Institute for Food Science, Kyoto University, for their kind advice and helpful support.

The author wishes to express great thanks to Dr. Ken-ichi Tanaka, Mr. Manabu Oki and Dr. Takao Matsuura, Kyoto Red Cross Blood Center, for their kindly supplying human platelets.

The author wishes to express his gratitude to Dr. Yukako Hayashi, Dr. Sakiyo Koseki, Mr. Kazuteru Wada and all the members of the laboratory of Protein Food, the Research Institute for Food Science, Kyoto University, for their helpful support and assistance.

Acknowledgement is made to Dr. T. Takenawa, Professor of the Institute of Medical Science, University of Tokyo, for the immunological experiments with PLC-I and II using anti-PLC antibodies.

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